THE INDUCED CHEMICAL DEFENSES OF NORWAY SPRUCE [*PICEA ABIES* (L) KARST.]: ECOLOGICAL FUNCTION AND REGULATION

Dissertation

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ABBREVIATIONS

ANOVA	analysis of variance
df	degree of freedom
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulphoxide
DOXP	1-deoxy-D-xylulose 5-phosphate
dw	dry weight
EDM	Embryo Development Medium
EGM	Embryo Germination Medium
FPP	Farnesyl Pyrophosphate (Farnesyl Diphosphate)
fw	fresh weight
GC-MS	Gas Chromatography – Mass Spectroscopy
GGPP	Geranyl Geranyl Pyrophosphate (Geranyl Geranyl Diphosphate)
GLM	General Linear Models
GPP	Geranyl Pyrophosphate (Geranyl Diphosphate)
HPLC	High Performance Liquid Chromatography
IPP	Isopenthyl Pyrophosphate
JA	Jasmonic Acid
LSD test	Least Significant Difference test
mAU	milli-absorption units
MEP	2-c-methyl-D-erythritol-4-phosphate
MJ	Methyl Jasmonate
MVA	Mevalonic acid
N	number
Pa	*Picea abies*
PP cells	Polyphenolic parenchyma cells
SD	Standard deviation
SE	Standard error
TD	Traumatic duct
TPS	Terpene synthase
UV	ultraviolet

INTRODUCTION

1.1. Plants as organic chemists *par excellence*

In natural habitats, plants are surrounded by an enormous number of potential enemies. Nearly all ecosystems contain a wide variety of bacteria, viruses, fungi, insects, mammals, and other herbivorous animals. Plants cannot avoid these potential pests and pathogens by moving away; they must protect themselves in other ways. In spite of their silent and sessile life, they are dynamic organisms that use chemical warfare to defend themselves. As a consequence, plants are capable of synthesizing a vast number of complex molecules, often known as "secondary" metabolites, that are believed to protect against herbivores and pathogens. Plants are unsurpassed as organic chemists and we can only stand back and admire their panoply of synthetic skills and virtuosity (Haslam, 1999). The immense number of structurally diverse compounds produced is perhaps the most striking feature of plant secondary metabolism. Each of the major groups of these metabolites, such as terpenoids, alkaloids and phenylpropanoids, includes several thousand different compounds identified to date (Croteau et al., 2000). In contrast to primary metabolites, they vary in their distribution in the plant kingdom and often occur only in specific taxa. Secondary metabolites therefore are thought to represent adaptive characters that have been subjected to natural selection during evolution (Wink, 2003). Some of the compounds have powerful biological effects, which have been known to man for thousands years. They accumulate in specialized organs such as resin ducts or glandular trichomes and, when released, act as (i) deterrents against pathogens or herbivores or are directly toxic to them; (ii) wound sealants; or (iii) relatively non-polar solvents for higher molecular-weight defensive compounds that would otherwise not go into solution (Gershenzon, 2002). However, the precise function of the vast majority of secondary metabolites is still uninvestigated.

1.2. Keeping the world green: chemical defenses in conifers

Most of our understanding of plant defense, especially chemical defense, has been obtained through studies on herbaceous species, especially the model plants *Arabidopsis thaliana*, tomato, maize and rice (Heath and Boller, 2002). Much less is known about the types of defenses employed by woody plants. These are long-lived and large organisms that live in dense stands where large numbers of herbivores and pathogens may build up over time. Moreover, because of their different life history than herbaceous plants (Bryant et al., 1991), they may be subject to qualitatively different patterns of herbivore and pathogen pressure. Among the dominant groups of woody plants, oncifers are the oldest having evolved over 300 million years ago during the carboniferous era, probably from a single ancestor (Steward and Rothwell, 1993). They are a distinctive and widespread group whose 500-600 species include some of the largest and longest-lived representatives of the plant kingdom (Kubitzki, 1990; Doyle, 1998). Of the 3 870 million ha of world's forest, about one-third is coniferous (FAO, 2005). In addition, conifers have unique tissues, such as those resulting from secondary growth of the stem, and so may require different modes of protection. As a consequence, conifers appear to have developed a large array of defensive attributes that potentially could protect them against pathogens or herbivores (Rosenthal and Janzen, 1979; Whitham et al., 1991; Phillips and Croteau, 1999; Walling, 2000; Degenhardt et al., 2003), including both constitutive and inducible defense systems (Berryman, 1972; Phillips and Croteau, 1999; Franceschi et al., 2005).

A major constitutive defense of particular importance to conifers is resin, a complex mixture of monoterpenes, sesquiterpenes and diterpenoid acids. Resin is stored in radial resin ducts derived from radial rays, axial resin ducts or canals, resin blisters, and resin cells (Franceschi et al., 2005). These structures accumulate resin internally under pressure. The preformed resin system is often the first defensive component encountered by organisms invading conifers. Upon damage by wounding or by an invading organism, the pressurized resin is released; its toxic nature can repel or flush the organism out of the bark, entrap the organism in sticky resin, or otherwise kill the invader (Berryman, 1972). Resin flow from radial phloem ducts can be enhanced by their connection to constitutive or induced axial resin ducts in the xylem (Christiansen et al., 1999; Nagy et al., 2000). Whereas resin-producing structures are found in all Pinaceae, they do not occur

constitutively in the secondary phloem of other conifer taxa (Hudgins et al., 2003a, 2004). Even in the Pinaceae, species of *Pinus* have well-developed resin duct systems, but other genera (e.g. *Abies, Tsuga,* or *Cedrus*) do not have preformed resin ducts (Bannan, 1936; Berryman, 1972). Other constitutive defenses include lignified cells and calcium oxalate crystals, all of which may create formidable physical and chemical barriers to attack (Wainhouse et al., 1990; Krekling et al., 2000; Hudgins et al., 2003b; Franceschi et al., 2005).

Because the biosynthesis of resin and other constitutive defenses is an energy-demanding process, plants have also developed defenses that are inducible upon damage by wounding or by an invading organism and that require expenditures of energy only when the plant is attacked. For conifers, inducible defenses are diverse and include structural changes and the synthesis of chemical agents, including terpenoids, phenolics, PR proteins and hydrolytic enzymes (Franceschi et al., 2005). Many induced defenses seem especially targeted towards pathogens and involve cellular and biochemical changes at the affected site that precede pathogen growth and tend to confine colonization to a discrete area (Berryman, 1972; Paine et al, 1997; Franceschi et al., 2005).

During and following herbivore or pathogen attack, resin flow can be quite extensive, especially in members of the Pinaceae. Part of this resin is from that stored in existing resin-producing structures, and there is evidence that the constitutive ducts can be activated to produce more resin (Ruel et al, 1998; Lombardero et al., 2000). Within 2–3 weeks after attack, new resin ducts, referred to as traumatic resin ducts, can also be induced to form (Alfaro, 1995; McKay et al., 2003). These ducts form above and below a damaged site or induced point on the stem (Franceschi et al., 2000; Nagy et al., 2000; Krekling et al., 2004). These ducts are interconnected with the radial resin ducts of the phloem (Nagy et al., 2000). The resin formed by traumatic ducts can be different than constitutive resin (Nault and Alfaro, 2001; Martin et al., 2002; Miller et al., 2005).

Protein-based chemical defenses in trees may be targeted towards specific enemies. For example, chitinases have been reported to be induced by pathogen attack and wounding in both *Picea abies* and *Pinus elliottii* (Sharma et al., 1993; Davis et al., 2002; Hietala et al., 2004; Nagy et al., 2004b), and these are presumed to be effective at hydrolyzing the cell walls of particular pathogens.

Inducible defenses that are activated upon initial attack include also the swelling and proliferation of polyphenolic parenchyma cells (PP cells) in the bark (Franceschi et al., 2000; Krokene et al., 2003), accompanied by changes in phenolic content (Brignolas et al., 1995a, 1995b, 1998; Evensen et al., 2000; Viiri et al., 2001; Lieutier et al., 2003). These multiple overlapping defense structures and systems may provide a formidable defense against a wide range of possible attacking organisms.

1.3. *Picea abies* (L) Karst., ecology, distribution, importance

Picea abies (Norway spruce) is the most abundant and economically important conifer species in northern and central Europe (Schmidt-Vogt, 1987). The species belongs to the genus *Picea,* which includes up to 35 species (Aldén, 1987), and is distributed over the cooler regions of the northern hemisphere, right up to the Arctic circle. *Picea* is a member of the sub-Division *Gymnospermae*, Class *Coniferopsida*, Order *Coniferae*, family *Pinaceae* (Kubitzki, 1990). Spruces are significant climax species, dominating most of the major forest ecosystems of Europe, Asia, and North America. In northern Europe, *Picea sitchensis* (Sitka spruce) and *Picea abies* (Norway spruce) each account for over 10% of total productive forestry (FAO, 2005). Norway spruce, although adapted to the cooler Northern climate, has "diversity centers" in southern Euorpe, which may have been its refugia during the last Ice Age (Scotti et al., 2000). *Picea abies* is a forest tree with a boreal and montane distribution extending south to northern Albania. It is presently distributed over the north-eastern European boreal forest with disjunct locations in the central European mountains (Figure 1.1) (Skrøppa, 2003).

The present-day range of Norway spruce is the result of post-Ice Age advances from at least two refugia: (i) a rapid early Holocene advance that spread out of Byelorussia and northern Russia and (ii) a mid- to late-Holocene front-like spread at high population densities moving from east to west into the Baltic republics and Finland, into northern Scandinavia, and then south and west toward its current distributional limits (Huntley and Birks, 1983; Giesecke and Bennett, 2004). The species has been estimated to be spreading out from its glacial refugia at 1000–2000 m/year (Scotti et al., 2000).

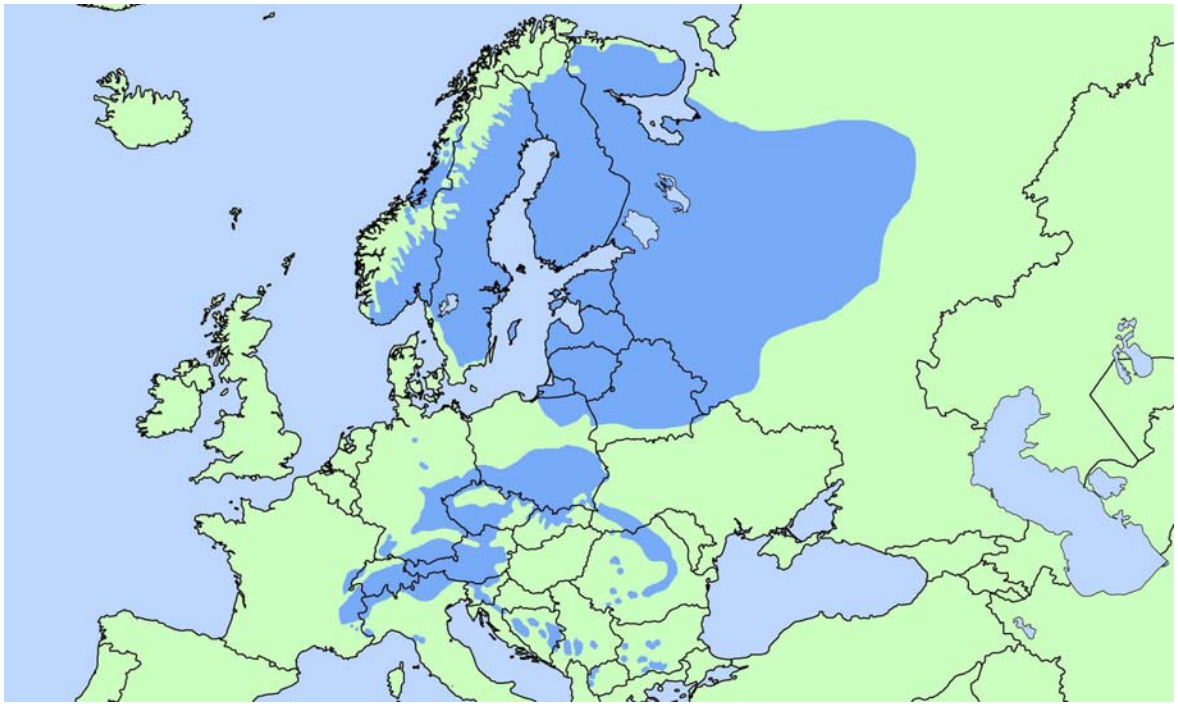

Figure 1.1. Natural distribution of *Picea abies* (L) Karst. (after Skrøppa, 2003).

Although it is native to central and northern Europe, Norway spruce is found throughout much of the United States and Canada (Gilman and Watson, 1994). It is found as an ornamental tree in urban environments, a windbreak and snowbreak in both urban and rural areas, and in pure stands for future harvest in forests. It is so common, widespread, vigorous, and healthy that most people do not realize that it is not a native of North America, in spite of its common name (Gilman and Watson, 1994).

1.4. The biology and ecology of bark beetles and white pine weevils

Bark beetles and pine weevils, known also as borers, attack conifers and are serious pests of forests or plantations (S.L. Wood, 1982; Mitchell et al., 1990). The larval stages of these insects tunnel beneath the bark and may cause enough damage to severely stunt or kill the tree. As the larvae tunnel in the conductive tissue of the tree, they interfere with sap flow and destroy the cambium (S.L. Wood, 1982). For many species, reproduction is contingent on the death of the tree (Berryman, 1972; Raffa and Berryman, 1983). Failure

to kill all or part of the tree usually results in failure to reproduce (Raffa and Berryman, 1983; Raffa and Berryman, 1987).

The spruce bark beetle (*Ips typographus*) (Coleoptera, Scolytidae) is considered the single most destructive of the bark beetles that inhabit the coniferous forests of the Palaearctic region (Berryman, 1972; D.L. Wood, 1982). A keystone species, it causes both small-scale and large-scale disturbances, thus driving forest succession in Eurasia (Christiansen and Bakke, 1988). The last outbreak (1971–1981) killed the equivalent of 5 million m^3 of spruce timber within a 140,000 km^2 area of southeastern Norway (Bakke, 1989). The genetic structure of bark beetle populations may have been influenced by their history since the last Ice Age, ca. 10 000 B.C. (Stauffer et al., 1999), and it is likely that *I. typographus* had the same refugial areas as the host tree*, Picea abies* (Stauffer et al., 1999). At low population densities this species breeds in the newly dead wood of Norway spruce [*Picea abies* (L.) Karst.], while at high population densities it may colonize living trees (Weslien et al., 1989). The initial phase of host selection is performed by individual beetles that arrest in response to visual (Strom et al., 1999) or chemical (Borden, 1984; Byers et al., 1988) cues, and subsequently either enter or depart from the host based on short-range chemicals and tactile stimuli (Moeck et al., 1981; Raffa and Berryman, 1983). If the host is acceptable, a male bores through the outer bark into the phloem tissue where it excavates a nuptial chamber and produces aggregation pheromones that attract females (D.L. Wood, 1982).

Another example of a serious pest of conifers is the white pine weevil (*Pissodes strobi* Peck, Coleoptera: Curculionidae). Particularly susceptible are plantations or nearby regenerating areas of white pine (*Pinus strobes* L.) in areas such as eastern Canada (Boucher et al., 2001a), while in western Canada, areas occupied by white spruce (*Picea glauca* Moench. Voss) and Sitka spruce (*Picea sitchensis* Bong. Carr.) are vulnerable (Alfaro, 1998). Eggs are laid by sexually mature female weevils in single holes drilled into the terminal leaders of suitable trees in early spring; the larvae which are hidden from sight, proceed to eat the phloem and soft cortex of the growing leaders, girdling and killing them (Boucher et al., 2001a; Sahota et al., 1998). High rates of egg laying on affected leaders support high rates of adult emergence (Boucher et al., 2001a). After feeding for 5 to 6 weeks, the larvae construct pupal chambers in the wood or pith of the terminal shoots,

and cover themselves with shredded wood and bark. New adults leave the tree by late summer and feed before overwintering (Mitchell et al., 1990).

The white pine weevil does not usually kill the trees, but does result in permanently deformed stem and crown, which devalue the affected trees and may slow their growth, especially after repeated attacks (Alfaro and Borden, 1985; Boucher et al., 2001a,b). Weevil attack can reduce growth by as much as 40% in heavily attacked stands in high hazard zones. The most severe attacks occur in relatively open, fast-growing stands that are 10 to 30 years old and between 2 and 20 m tall, usually just those stands that have received considerable attention from foresters to encourage maximum growth. The weevil begins to attack spruce plantations when they are about five years old (Alfaro, 1998). The cryptic development of the larvae, in addition to the initial random attack of the adults, makes the white pine weevil an especially difficult pest to control.

1.4.1. “The Dream Team." Bark beetle and blue-stain fungi: Multiple attacks against multiple defenses

The spruce bark beetle that colonizes living conifers is frequently associated with specific fungi that are carried in specialized structures or on the body surface (Paine et al., 1997). These fungi are introduced into the tree during the attack process. The relationship between beetles and blue-staining fungi has been described as symbiotic or mutualistic (Whitney, 1982). The fungal species may benefit from the association with the beetles by being transported to new host trees. Beetle species may benefit from association with fungi (i) by feeding on the fungi or (ii) by the fungi contributing to the death of the host trees through mycelial penetration of host tissue, toxin release, interactions with preformed and induced conifer defenses, or the combined action of both beetles and fungi during colonization (Paine et al., 1997).

The most virulent fungal pathogen associated with *I. typographus* is the blue-staining fungus *Ceratocystis polonica* (Siem.) C. Moreau (Krokene and Solheim, 1998). The genus *Ceratocystis* Ellis & Halsted, which sensu stricto represents a relatively small group of plant parasites, occurs primarily on angiosperms (Kile, 1993). The fungus invades the vascular system and when inoculated into healthy trees, *C. polonica* induces extensive lesions (Christiansen and Solheim, 1990; Solheim, 1993). The population dynamics of *I.*

typographus have a strong influence on the incidence and frequency of *C. polonica*, as the fungus occurs less frequently during endemic periods but becomes more frequent during outbreaks of the insect (Solheim, 1992). The fungus colonizes the living sapwood of wounded trees and is an important symbiont of bark beetles. Although the mechanisms are not fully understood, the most comprehensive theories suggest that trees are killed as a result of the simultaneous action and interaction of both fungus and bettle rather than successive actions of the two (Christiansen and Solheim, 1990; Harrington, 1993; Solheim, 1993; Harrington and Wingfiled, 1998). *C. polonica* is also economically important in that it is capable of staining sapwood blue-gray.

1.5. Terpenoids and phenolics

The epithet "secondary" usually connotes an ecological function for a metabolite. Among the secondary metabolites, terpenoids and phenolic compounds are among the most important and the best-studied defensive chemicals.

1.5.1. Terpenoids: biosynthetic pathways and cellular compartmentation

The best studied chemical defense of *Picea abies* and other conifers is the oleoresin found in foliage, stems and other organs, a defense system that has existed for at least 50 million years (Labandeira et al., 2001). Oleoresin is composed largely of terpenes, the largest class of plant secondary compounds (Gershenzon and Kreis, 1999). The term terpenoid or terpene is derived from the German word for turpentine, *Terpentin*, from which the first members of this group of chemicals were isolated and their structures determined (Croteau, 1998). The work on terpenes from conifers started almost a century ago, with the pioneering book "*Die Harze und die Harzebehälter"* (Tschirch, 1906).

Two biosynthetic pathways lead to the formation of the basic structural unit of terpenoid synthesis (Gershenzon and Kreis, 1999) (Figure 1.2). In the classical mevalonic acid (MVA) or mevalonate pathway, three molecules of acetyl coenzyme A are linked (pyro)phosphorylated, decarboxylated, and dehydrated to yield isopentenyl pyrophosphate (IPP). In the alternate pathway, also referred to as the DOXP or DXP pathway, the MEP pathway, and sometimes as the nonmevalonic or mevalonate - independent pathway, 3-

phosphoglycerate (3-PGA) and two carbon atoms derived from pyruvate apparently combine to generate a first intermediate, 1-deoxy-D-xylulose 5-phosphate (DOXP or DXP), then 2-C-methyl-D-erythritol 4-phosphate (MEP), which eventually is converted to IPP (Lichtenthaler, 1999; Lange et al., 2000).

Most terpenoids are formed from "head-to-tail" condensations of branched five-carbon isoprene units and are classified according to the number of five-carbon units present in the carbon skeleton. IPP and its isomer, dimethylallyl diphosphate (DMAPP), are the actual five-carbon building blocks for the formation of larger terpenoid molecules (Figure 1.2). DMAPP serves as a primer to which IPP units can be added in sequential chain-elongation steps. These reactions, catalyzed by prenyltransferase enzymes, connect isoprene units to one another.

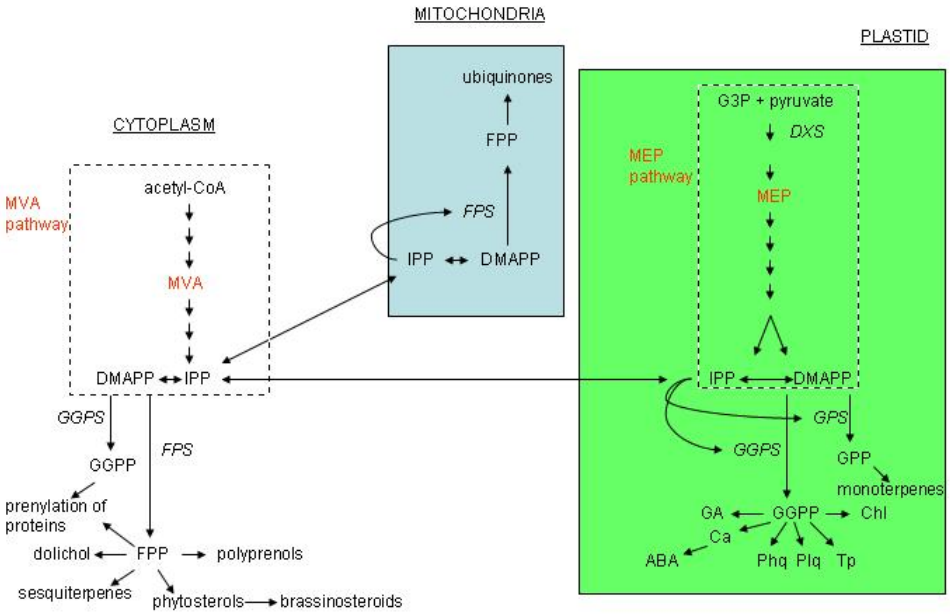

Figure 1.2. Organization of terpene biosynthesis in plants. (DMADP - dimethylallyl diphosphate, IDP -isopentenyl diphosphate) (modified after Gershenshon and Kreis, 1999).

Thus IPP and DMAPP combine to form a C_{10} precursor (geranyl diphosphate, GPP) for all 10-carbon compounds, called monoterpenes. The addition of another molecule of IPP yields a C_{15} precursor (farnesyl diphosphate, FPP) for all 15-carbon isoprenoids, called

sesquiterpenes. The structural diversity of sesquiterpenes greatly exceeds that of monoterpenes, because many more types of cyclization can occur in a precursor with five additional carbon atoms (Joulain and König, 1998). Mono- and sesquiterpenes generally are volatile, making the resin fluid as well as acting as plasticizers for the more viscous components.The addition of three molecules of IPP to DMAPP gives the C_{20} precursor (geranylgeranyl diphosphate, GGPP) of the diterpenes. More than 3000 diterpene structures have been defined, usually bearing a variety of oxygen-containing functional groups. Diterpene acids are particularly important in resin (Langenheim, 2003).

The two different pathways to IPP appear to be compartmentalized (Newman and Chappell, 1999), within plastids, mitochondria, and cytosol-endoplasmic reticulum. Each compartment produces different products. The mevalonate pathway operates in the cytosol-ER compartment to produce sesqui- and triterpenes, whereas the alternative DXP pathway operates in plastids to produce mono- and diterpenes (Figure 1.2) (Gershenzon and Kreis, 1999). Compartmentation is significant in regulating terpenoid synthesis because it allows independent control of different branches of the pathway at different sites in the cell. Within a compartment, metabolic dynamics depend on the kind of enzymes present and the permeability of intercellular membranes to precursors, intermediates, and products. The differentiation of secretory structures may also provide control over terpenoid production since specialized secretory structures are apparently required for the synthesis of many plant mono-, sesqui-, and diterpenes, including those found in resin, (Gershenzon and Croteau, 1990; Gershenzon and Croteau, 1993).

The three prenyl diphosphates, GPP, FPP, and GGPP, are the substrates of a large family of terpene synthase enzymes (TPS) that catalyze the formation of an amazing variety of parent skeletons of monoterpenoids, sesquiterpenoids and diterpenoids (Bohlmann et al., 1998a; Huber et al., 2004; Martin and Bohlmann, 2005). TPS utilize an electrophilic reaction mechanism assisted by divalent metal ion cofactors. Monoterpene synthases from conifers are operationally soluble proteins with native (monomeric) sizes in the 50–70 kDa range, pH optima in the 6.8 to 7.8 range, and most are associated with plastids *in vivo* (Bohlmann et al., 1997; Bohlmann et al., 1998a).

The large number of terpenoids present in conifers is due on the one hand to the large number of different TPS enzymes in any given conifer species. From the molecular studies

it is known that grand fir, *Abies grandis*, for example, contains at least seven mono-TPS, three sesqui-TPS, and one di-TPS (Bohlmann et al., 1997, 1998a,b; Steele et al., 1998a,b). Three different mono-TPS and one sesqui-TPS gene have been characterized from loblolly pine, *Pinus taeda* (Phillips et al., 2003). Similarly, Norway spruce, *Picea abies*, contains a large family of diverse TPS of which five mono-TPSand three sesqui-TPS are known (Fäldt et al., 2003; Martin et al., 2004). The variety of terpenes present is also due to the fact that many conifer tepene synthases produce multiple products. In Norway spruce, i.e. a monoterpene synthase (PaTPS-Lim) is shown to produce 87.8% (-)-limonene, 5.2% myrcene, 4.4% (-)-α-pinene, 2.1% (+)-limonene, and 0.5% (-)-β-pinene (Martin et al., 2004), while a sesquiterpene synthase (PaTPS-Lon) is shown to produce 60.6% longifolene, 5.9% longicyclene, 3.4% E-β-farnesene, 2.1% longiborneol, 1.3% cyclosativene, 1.4% β-longipinene and some other sesquiterpenes each less than 1.4 % (Martin et al., 2004).

TPS genes may be differentially expressed prior to, during, and following attack by insects or pathogens and the enzymes that produce terpenes often differ between constitutive resin (performed resin stored in secretory structures) and induced resin (that synthesized at the side of an injury) (McKay et al., 2003; Miller et al., 2005; Steele et al., 1998a,b). In general properties, however, the constitutive and inducible synthases are indistinguishable (Trapp and Croteau, 2001).

1.5.2. Phenolic compounds

Another large group of plant secondary metabolites that often have defensive roles is the phenolics (Nicholson and Hammerschmidt, 1992). They display great structural heterogeneity and are generally characterized as aromatic metabolites that possess or once possessed one or more “acidic” group attached to the aromatic arene (phenyl) ring (Herrmann and Weaver, 1999). Plant phenolics are biosynthesized by several different routes and thus constitute a heterogeneous group from a metabolic point of view. Two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway. The shikimic acid pathway participates in the biosynthesis of most plant phenolics. The malonic acid pathway, although an important source of phenolic secondary products in

fungi and bacteria, is of less significance in higher plants (Hahlbrock and Scheel, 1989). Phenolic compounds are highly compartmentalized. They usually accumulate in the central vacuoles of guard cells and epidermal cells as well as in the subepidermal cells of leaves and shoots, or are covalently linked to plant cell walls (Strack et al., 1988; Schnitzler et al., 1996; Hutzler et al., 1998).

This structurally diverse and ubiquitous group of plant compounds has been suggested to play a variety of roles in plant defense against pathogens as phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, and activators of plant defense genes (Hammerschmidt, 2005). In Norway spruce, phenolic compounds are thought to work together with the terpene oleoresin to provide a defensive barrier after the bark surface is breached (Woodward and Pearce, 1988a,b). In recent years, a number of phenolic compounds have been identified in spruce bark, including stilbenes, flavonoids, and tannins (Pan and Lundgren, 1985; Solhaug, 1990; Toscano-Underwood and Pearce, 1991a, b). Trees with a high phenolic biosynthetic capacity and high phenolic diversity are hypothesized to be more resistant than trees with a low biosynthetic capacity that produce only one type of phenolic compound (Lieutier et al., 2003). Moreover, recently it has been shown that wounding induced changes in certain cells believed to produce phenolic compounds in the *Picea abies* stems (Franceschi et al., 1998; Franceschi et al., 2000; Krekling et al., 2000; Krekling et al., 2004; Nagy et al., 2004a). These cells found in the secondary phloem are referred to as polyphenolic parenchyma (PP) cells. They occur in concentric rings, 1-2 cells thick, surrounded by sieve cells. One ring of PP cells is formed per year (Krekling et al., 2000). The vacuoles of these cells harbor a material that appears to be phenolic based on its intense fluorescence under 450-490 nm light (Franceschi et al., 1998) and strong staining with the periodic acid-Schiff procedure (Franceschi et al., 2000). In addition, phenylalanine ammonia lyase, a major enzyme in plant phenolic formation, has been localized to the PP cells by immunolocalization (Franceschi et al., 1998). Upon wounding or fungal infection, the PP cells increase in size with a strong increase in periodic acid-Schiff's staining, and the phenolic material appears to be released to the wall of surrounding cells (Franceschi et al., 2000; Krekling et al., 2004).

Several studies have looked for changes in phenolic quantity and composition after wounding or fungal infection (Brignolas et al., 1995b, 1998; Evensen et al., 2000; Lieutier

et al., 2003). However, the changes observed were either unremarkable (increase or decrease of 2-fold or less) or poorly replicated. Thus, it is still not clear what changes in phenolic chemistry are associated with the dramatic changes observed in the anatomy of the PP cells.

1.6. Mechanism of defense induction and signaling

The induction of chemical defenses following pathogen or herbivore attack requires mechanisms for sensing the presence of an enermy and then rapidly triggering the synthesis of protective metabolites. Though the exact mechanisms remain unclear, herbivore or pathogen-produced substances called elicitors are thought to induce the signal transduction pathways in the plant, which leads to the activation of genes of biosynthetic pathways involved in defense. Three major pathways are thought to be activated: 1) the octadecanoid pathway with jasmonic acid as the key compound, 2) the shikimic acid pathway with salicylic acid as a key compound, and 3) the ethylene pathway (Dong, 1998; Kunkel and Brooks, 2002). Deployment of defenses is energetically costly, so a trade-off between the activation of resistance to a particular pest or pathogen and down-regulation of other defenses is common. Conversely, activation of broad-range resistance in response to an initial attack may serve to deter opportunistic agents. Thus, the interaction among jasmonic acid, salicylic acid, and ethylene defense signaling pathways can be antagonistic, cooperative, or synergistic, depending on the plant species, the combination of organisms attacking the plants, and the developmental and physiological state of the plant (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Thaler et al., 2002; Rojo et al., 2003). The cross-talk between signaling pathways adds plasticity to the defense response, allowing the plant to adjust the defense depending on the combination of stimuli present. This has been proposed to be central to the plant's ability to fine-tune the induction of plant defenses in response to different plant pests and pathogens (Kunkel and Brooks, 2002). Although all three pathways might be activated, the octadecanoid pathway (Figure 1.3) is thought to play the most important role in signal transduction. The cascade starts with the conversion of 12-oxo-linoleic acid to 12-oxo-phytodienoic acid (OPDA) via the intermediates (13*S*)-

hydroperoxylinolenic acid and allene oxide. OPDA is reduced and degraded by three β-oxidation cycles to epijasmonic acid and jasmonic acid (Figure 1.3).

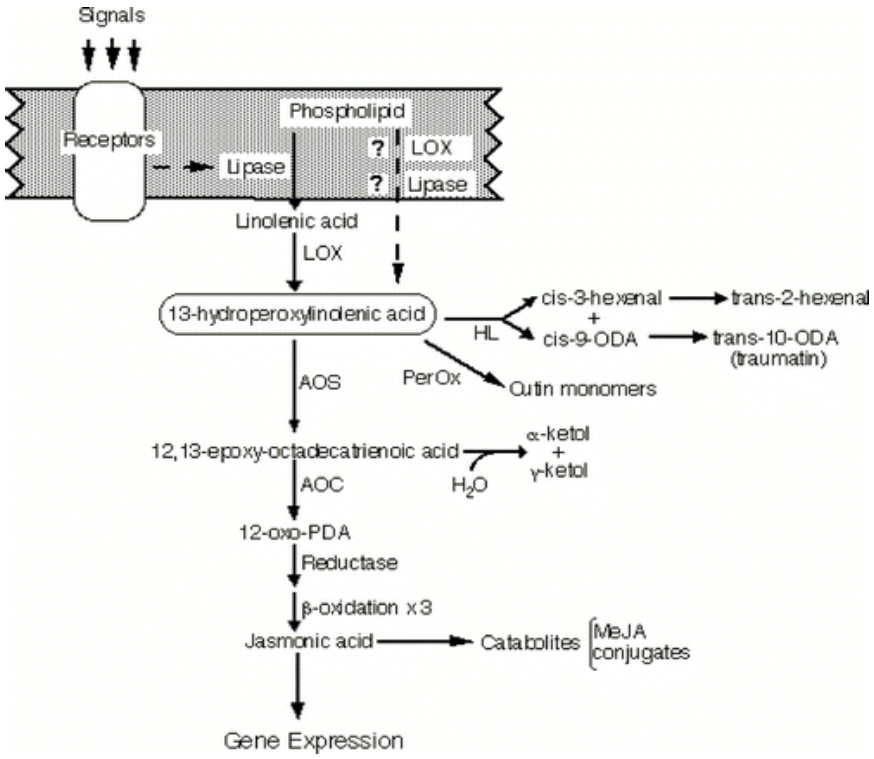

Figure 1.3. Signal transduction via the octadecanoid pathway and the formation of the jasmonic acid. (AOS - allene oxide synthase, AOC - allene oxide cyclase, OPDA - 12-oxo-phytodienoic acid) (modified after Stratmann, 2003).

How the signals regulate the expression of defense genes is unknown. Although different stimuli activate different signaling pathway and defense-signaling pathways are not linear (Rojo et al., 2003), the octadecanoid pathway seems to play the most important role in induced defense, especially in of the long-distance variety (Stratmann, 2003).

1.7. Methyl jasmonate induces defense responses

Research on fatty acid-based signaling systems in plants has focused mainly on the hormonally active compound, jasmonic acid (JA) and a rapidly growing body of literature indicates that jasmonic acid is involved in induction of a wide range of plant defenses (Regvar et al., 1997; Dicke et al., 1999; Howe, 2001; Babst et al., 2005). In contrast, its

methyl ester (methyl jasmonate, MJ) although widely distributed in the plant kingdom (Creelman and Mullet, 1997), has not often been shown to have an endogenous role in controlling plant defense. The concentrations of both compounds are tightly regulated in plants (Stintzi, et al., 2001); the relative basal amount of each jasmonate family member may differ from tissue to tissue (Blechert et al., 1995). Methyl jasmonate was first identified as a component of the essential oil of several plant species, while jasmonic acid was first obtained from a fungal culture filtrate (Creelman and Mullet, 1997; Beale and Ward, 1998).

Considered for a long time only as plant hormones that can promote senescence and act as growth regulators (Creelman and Mullet, 1997), JA and MJ have been shown by subsequent research to play other physiological roles. Thus, jasmonates are shown to be involved in metabolic regulation (Ozawa et al., 2000; Van der Fits and Memelink, 2000), and semiochemical communication (Birkett et al., 2000). It has been shown that JA specifically alters gene expression and that wounding and elicitors could cause JA/MJ accumulation in plants, implying jasmonates play a role in plant defense (Beale and Ward, 1998; Liechti and Farmer, 2002; Turner et al., 2002). The jasmonate response is known to protect plants against a wide range of insect herbivores and pathogens (Beale and Ward, 1998; Thaler, 1999; Thaler et al., 2004).

In conifers, jasmonates have been shown to promote the formation of an oxygenated sesquiterpene, todomatuic acid, and an oxygenated diterpene, paclitaxel (taxol), in cell cultures (Bohlmann et al., 1998b; Ketchum et al., 1999). Methyl jasmonate is known to increase terpene levels in conifers when applied to saplings (Martin et al., 2002, 2003) or cell suspension cultures (Yukimune et al., 1996; Ketchum et al., 1999), as well as to increase the resistance of *P. abies* seedlings to the root pathogen *Pythium ultimum* (Kozlowski et al., 1999). However, it is not known if methyl jasmonate can increase resistance to stem pathogens or herbivores of conifers. In addition, no detailed information is available on the effect of methyl jasmonate on the terpene content of mature trees, and the effect of this compound on other potential defenses, such as phenolic compounds, has not been investigated.

1.8. The culture of conifer cells as a system for studying the production of defense compounds

Conifers are difficult organisms for biochemical studies because of their large size and many types of specialized tissues which make it hard to study an individual pathway, such as terpene biosynthesis, that is confined to specialized tissue. These problems are compounded by the lack of modern molecular and genetic tools. In comparison to Arabidopsis, for example, conifers have long generation times, large genomes, a lack of well-defined mutants and difficulties in transformation. Thus, the use of plant cell culture is an attractive alternative system for studying the production of conifer defense metabolites. In conifers, for example, cell cultures have been used to study the levels of ethylene production, chitinase activity, and glucanase activity in cells of loblolly pine (*Pinus taeda* L.) (Popp et al., 1996). In Scots pine (*Pinus sylvestris* L), cell cultures were used to investigate the formation of free and cell-wall-bound stilbenes and the production of chitinases (Lange et al., 1994; Pirttila et al., 2002), while in *Picea abies*, the interaction between the oxidative burst, salicylic acid and the role of calcium as secondary messenger was elucidated with cultures (Messner and Schroder, 1999).

Cell cultures have been established from many plants but often they do not produce sufficient amounts of the required secondary metabolites. However, in some cases the production of secondary metabolites can be enhanced by the treatment of the undifferentiated cells with elicitors such as methyl jasmonate, salicylic acid, cell wall constituents of various microorganisms, or enzymes such as pectinase or cellulose (Ebel and Cosio, 1994). In conifers, jasmonate treatment has been applied to the suspension-cultured cells of a few gymnosperms (Mueller et al., 1993). A very rapid accumulation of the diterpene paclitaxel (taxol) in a plant cell culture has been achieved by optimizing the concentration of jasmonates and the timing of their application (Ketchum et al., 1999). New insights into the biosynthesis and regulation of monoterpenes in *Cupressus lusitanica* cell cultures are provided by the work of Zhao and Sakai (2003) and Zhao et al. (2006). It has been shown for instance that *C. lusitanica* regulates biosynthesis of defensive secondary metabolites by using toxic *β*-thujaplicin as the first phase of defense and other monoterpenes as a secondary one (Zhao et al., 2006). Thus, plant cell suspension cultures can be useful tools for investigating terpenoid biosynthesis allowing the elucidation of the

biosynthetic pathway and determination of the factors that regulate terpene formation. In this study, *P. abies* cell suspension cultures were used to study the regulation of induced terpene defenses in this species.

1.9. Purpose and objectives of the study

The epithet "secondary" connotes an ecological function for a metabolite. The terpenoid resin and phenolic constituents have been implicated as "secondary" metabolites that play a role in protecting trees against bark beetles and other enemies, but it has been difficult to prove these defensive roles under natural conditions. To rigorously establish an ecological function, one must manipulate the expression of a metabolite and verify that an ecological interaction is subsequently altered. Such manipulative experiments are possible with conventional techniques when metabolites are externalized, but for plants that store metabolites internally in a highly tissue-specific manner, manipulation is only possible when specific regulatory compounds are available or when genes regulating secondary metabolite production can be altered (silenced or over-expressed). Unfortunately, the plant systems that are currently readily transformable such as *Arabidopsis thaliana* (L.) are frequently not optimal for ecological research.

In this study, I examined the induced chemical defenses of Norway spruce (*Picea abies*), defensive compounds whose levels increase following herbivore or pathogen attack. *Picea abies* was chosen as it is a significant climax species dominating most of the forest ecosystems northern and central Europe and known to produce copious amounts of isoprenoid products as a defense. The induction of several different classes of induced defenses in *Picea abies* were investigated, including terpene-containing resins and phenolic compounds. Methyl jasmonate, a well-known plant hormone and inducer of plant defense responses, was used to manipulate the biochemistry of mature *Picea abies* and test their resistance to attack by *I. typographus* (the spruce bark beetle), its associated pathogen, the blue-stained fungus *Ceratocystis polonica*, and to *Pissodes strobi* (white pine weevil). Studies with these pests were carried out with intact trees outdoors to achieve the most realistic experimental conditions possible.

My focus was on not only the defensive roles but also on the mechanisms and the pathways inloved in the spruce induced defense. Here I took the advantage of the embryogenic spruce liquid suspension cultures to model these defense responses in a system more amenable to regulatory studies.

MATERIALS AND METHODS

2.1. Effect of methyl jasmonate treatment on defense responses to the pathogen *Ceratocystis polonica*

2.1.1. Methyl jasmonate treatment and sampling

On 30-31 May 2001, six trees from each of six clones were selected from a plantation of 40-year-old Norway spruce clones at Hogsmark, Ås, SE Norway (diameter at 1.3 m above ground: 18.27 ± 2.11 cm; height: 18.39 ± 1.77 m [mean ± SD]). Three of these clones (75, 76, and 80) were half-sibs, whereas the others (63, 85, and 126) were unrelated. One tree per clone was randomly assigned to each of four different treatments with methyl jasmonate (MJ): 5, 25, 50, and 100 mM MJ in water with 0.1% Tween 20, or to a control with water and 0.1% Tween 20. Tween 20 helps to solubilize MJ in water and acts as a surfactant to help spread the solution evenly over the hydrophobic bark surface. The solutions were applied with a paint roller to the surface of a 1.2-m-high section of the stem from about 1.0 to 2.2 m above ground. The bark was kept wet for at least 5 minutes, and care was taken to prevent the liquid from running farther down the stem. During the two application days there was only a slight breeze, and maximum air temperatures of 10.3 and 19.9°C, respectively, were recorded at Ås Meteorological Station, ca. 5 km away from the experimental stand.

Four weeks after MJ treatment (27-28 June), all trees were mass-inoculated with *Ceratocystis polonica* (400 inoculations/m^2, on average 276 inoculations/tree) to assess tree resistance. Mass inoculations, evenly spaced over the MJ-treated stem area, were carried out by removing a bark plug with a 5-mm cork borer, inserting inoculum in the wound, and returning the plug to its original position. The inoculum consisted of actively growing mycelium of *C. polonica* (isolate no. NISK 93-208/115) cultured on malt agar (2% malt and 1.5% agar).

Samples of bark and sapwood for chemical analyses were taken from the control and MJ-treated trees on three occasions: (1) immediately before treatment with MJ or Tween 20 control, (2) immediately before fungal mass-inoculation, and (3) at the felling of

the trees, 15 weeks after mass inoculation. On the first occasion, three samples (20 × 20 mm), including bark, cambium, and outer annual rings of sapwood, were taken 10 cm apart, with the lowermost sample 40-50 cm above the upper edge of the stem section that was to be treated with MJ. On the second occasion, three samples were taken in a similar way, but inside the treated stem area. On the last occasion, one sample was taken within and another 50 cm above the treated area. Material for anatomical analysis (12 mm width × 15 mm height, including bark, cambium, and outer annual rings of sapwood) was collected on the second occasion only. Two samples were taken on opposite sides of the stem inside the treated area. In late July, the stems of all trees were inspected for resin on the surface of the treated area. Resin flow from inoculation points was visually assessed on a scale from 0 (no flow) to 5 (abundant flow from almost all points). Photos of the sections were taken to document the status. At felling, tree height was measured and two thin discs (5-10 mm) were cut 0.4 m inside the lower and upper edges of the inoculated sections on each tree.

Nine trees from a single clone (123, a half-sib to clone 126 that was used in 2001) were selected from the Hogsmark plantation on 24 June 2002. These trees were from a different clone than those used in 2001 since an insufficient number of individuals was available from previously studied clones to carry out further investigations in 2002. Three randomly selected trees of clone 123 were pretreated with 100 mM MJ in 0.1% Tween, three with Tween control, and three with 50 *C. polonica* inoculations/m^2. Samples of bark and sapwood for anatomical and biochemical analyses were taken at the time of pretreatment and 8 and 35 days afterwards.

2.1.2. Measurements of fungal growth, cambium necrosis, and anatomy

To quantify fungal colonization of host tissues, the proportion of the sapwood that had been blue-stained by the fungus and the proportion of dead cambium circumference were measured on the two stem discs that were removed from each tree, as described in Krokene and Solheim (1998). To quantify anatomically based defense reactions, samples containing bark and sapwood were prepared as described in Krokene et al. (2003) and examined with light microscopy. Briefly, samples were placed directly in fixative in the field, rinsed with buffer, dehydrated in a graded series of ethanol, and embedded in L. R.

White acrylic resin (TAAB Laboratories, Aldermason, Berkshire, UK). Cross-sections (1 μm thick) were cut on a diamond knife, dried onto gelatin-coated slides, stained with Stevenel's blue (Del Cerro et al., 1980), and mounted with immersion oil. Digital images were recorded at magnifications of 10× (phloem) and 4× (xylem) using a Leica DC300 CCD camera mounted on a Leitz Aristoplan photomicroscope, and analyzed using image analysis software (ImagePro Plus, ver. 3.0, Media Cybernetics, Leiden, The Netherlands). In the xylem, we measured the percentage of coverage of traumatic resin ducts (including the epithelial cells lining the ducts) across 1.1 mm in the tangential direction on each section. In the phloem we measured polyphenolic parenchyma cells (PP cells) in three annual rings of PP cells (1, 6, and 11 years old) in selected clones that later were found to respond strongly to methyl jasmonate treatment (clones 76, 80, 85). Cross-sectional areas of PP cells and the phenolic bodies inside these cells were determined as proportions relative to the total image area.

2.1.3. Statistical analyses

Data were subjected to ANOVA, using the general linear model (GLM) procedure of the SAS software package (SAS Institute, 1996). If treatments were significantly different ($P < 0.05$), means were separated by LSD at $P = 0.05$. Proportional data were arcsin-transformed before ANOVA to correct for unequal variance and departures from normality. Chemical data were analyzed using SPSS 10.1 for Windows™ (SPSS, Chicago, 2003). Pairs of means were compared using the paired-samples t-test, and means of two groups were tested using the independent-samples t-test. Multiple comparisons were done with one-way ANOVA, and linear associations were examined using simple correlation analyses.

2.2. Effect of methyl jasmonate on defense responses in *Picea abies* to the bark beetle *Ips typographus*

2.2.1. Effect of methyl jasmonate treatment on beetle colonization of live trees

The objective of this experiment was to investigate the effects of MJ treatment on beetle colonization of live trees. In the spring of 2003, twelve trees were randomly selected in an open, pure stand of mature Norway spruce (60 years old, tree height ca. 28 m, diameter at breast height 27.85 ± 3.09 (SD) cm). On 26 May, a stem section between 1.5 and 4.5 m above ground was divided into east- and west-facing halves by two vertical lines, using a water-based latex paint. One half of each tree was treated with 100 mM MJ and the other half was left untreated to serve as a control (MJ_C). MJ was sprayed onto the stem using a small spray gun, while carefully avoiding contaminating the control side. The sprayed bark was kept moist for at least 5 minutes. The weather was sunny with a slight breeze during application; hence the surface was completely dry within a few hours. Previous experiments have demonstrated that external MJ application induces anatomical defense reactions and increases tree resistance to the pathogenic fungal associates of *I. typographus* (Franceschi et al., 2002).

Three weeks after MJ application (16 June) four samples containing the bark and outermost sapwood (1.6 cm wide × 5 cm high × 1 cm deep) were removed for anatomical investigation from each tree at 1.5 and 3.5 m above ground, two on the treated side and two on the control side. At each site a smaller sample (1.6 × 1.6 × 1 cm) for analysis of terpenes and phenolics was removed, quickly frozen in liquid N_2, and transferred to a –80 ºC freezer. The anatomical samples were immediately placed in fixative (2% paraformaldehyde and 1.25% glutaraldehyde in 50 mM L-piperazine-N-N'-bis (2-ethane sulfonic) acid buffer, pH 7.2).

On 17 June, an Ipslure pheromone dispenser (Borregaard, Sarpsborg, Norway) was placed on each tree 2 m above ground to induce attack by *I. typographus*. The dispensers were placed on the north side of the trees on the border between the MJ-treated and untreated sides. Because the beetle population in the area was relatively low and the main flight of the beetles already had taken place, an additional Ipslure dispenser was added three days later to enhance attraction. Beetle aggregation remained moderate on all trees except one, which was located on the south-facing edge of the stand. Unlike the other 11 trees, which survived moderate attacks,

this tree was mass-attacked and killed by the beetles. The pheromone dispensers remained on the stems until 25 July, when the trees were sampled to assess the beetles' attack success.

For sampling, the outer cork bark was carefully shaved away on both sides of trees at the dispenser height. A transparent plastic sheet (210 × 297 mm) was placed on the stem within the shaved area, with the long side oriented vertically and well away from the dividing lines between the two treatments. All entrance holes covered by the sheet and penetrating into the live phloem were marked, and the more developed beetle galleries were traced. On 26 August, the trees were sampled again immediately above the first sampling site, using the same method. The tree that had been mass-attacked and killed was excluded from the experiment, since its entire bark was full of well-developed beetle galleries. In the laboratory we recorded the number of entrance holes and incipient galleries (i.e. tunnels longer than 10 mm), and total length of all maternal galleries on the plastic sheets. When multiple galleries extended from a single entrance hole, we recorded the sum of their lengths.

On 24 July 2003, 12 other Norway spruce trees in the same stand (diameter at breast height 28.59 ± 3.19 cm) were treated with MJ as described above to see if MJ treatment in one year would have any effect on beetle colonization the following year. Samples for anatomical and chemical analyses were removed from the trees the following spring (12 May 2004) as described above, and two days later the trees were baited with pheromone dispensers to induce attack by *I. typographus*. At this stage, there was extensive resin flow in some trees on bark that had been treated with MJ, and resin flow was assessed qualitatively on a scale from 0 (no resin) to 4 (extensive resin flow). On 16-17 June 2004, the outer bark was removed and the outcome of the beetle attacks was assessed as described above.

In both years, all anatomical samples were taken to the laboratory and processed as described in Krokene et al. (2003). Briefly, samples were rinsed with buffer, dehydrated in a graded series of ethanol, and embedded in acrylic resin. Cross-sections (1 μm thick) were cut on a diamond knife, dried onto gelatine-coated slides, stained with Stevenel's blue (Del Cerro et al., 1980), and mounted with immersion oil. Digital images were recorded at magnification of 5× using a Leica DC300 CCD camera mounted on a Leitz Aristoplan photomicroscope. The extent of TD formation in the xylem was quantified as the percentage of tracheid lanes that contained TDs (including the epithelial cells lining the ducts) across the full tangential width of each section (1380 μm).

2.2.2. Effect of methyl jasmonate treatment on beetle colonization, egg laying, and attraction of conspecifics

The objective of this experiment was to investigate effects of MJ treatment on beetle colonization, egg laying, and attraction of conspecifics. On 7 May 2004, a 3-m section of the lower stem of a 40-year-old spruce tree was treated in the same way as in experiment 1: half the bark circumference was treated with 100 mM MJ and the other half was left untreated. Three-and-a-half weeks later (1 June) the tree was felled and the 3-m stem section was cut into four bolts. An untreated ramet of the same clone was felled and sectioned the same way. The bolts were sealed at both ends with melted paraffin wax to minimize desiccation and cold-stored at 4 °C for later use.

Assay units consisted of fresh bark-phloem disks in large Petri dishes (150 mm diameter, 25 mm deep), as described by Erbilgin and Raffa (2000). Briefly, the phloem and outer bark were peeled off the bolts and cut into 150-mm-diameter circles using a hole saw (177 cm^2). The circles were then placed with the cambium facing downwards in the Petri dishes and melted paraffin wax was applied on the top and sides of the circle to minimize desiccation of the phloem. Three treatments were tested: (1) bark from the treated half of the MJ-treated tree (MJ), (2) bark from the untreated half of the same tree (MJ_C), and (3) bark from the untreated ramet (Control). Bark beetles were collected in traps baited with Ipslure pheromone dispensers and kept at 60-65% relative humidity and 4 °C for up to 10 days until use. Males and female *I. typographus* cannot be reliably distinguished based on morphology. However, traps with pheromone dispensers capture roughly equal amounts of each sex. To ensure that both sexes would be present in each assay unit, we introduced 10 beetles per unit. The Petri dishes were covered with mesh screen to prevent the beetles from escaping.

The beetles were allowed to colonize the bark disks for 24 hr at room temperature before the units were suspended from flight interception traps in the field. The traps consisted of two thin sheets of clear acrylic plastic (39 cm high × 21 cm wide) mounted vertically and crosswise over a plastic funnel (22 cm in diameter) fitted with a collecting bottle. One assay unit was placed as bait within the funnel, just below the plastic sheets. Fifteen traps were deployed in openings within a spruce stand, constituting five blocks in a randomized complete block design, with 15 m between treatments within a block and 50 m between blocks. The traps were placed with the collecting bottle hanging close to the top of the ground vegetation.

The assay units were replaced with fresh units (prepared from the cold-stored bolts) and re-randomized every 5 days over a period of 16 days (3-17 June 2004) to provide a total of 15 replicates per treatment.

After the 5-day trapping period the bark disc was dissected and the following data were recorded for each disc: number of attacks (male entrance holes), number of maternal galleries, total length of maternal galleries, and total number of egg niches. Only beetles that had tunnelled more than 10 mm were considered to be successful colonizers. Insects caught in the traps were identified as to species and sexed. We also sexed the beetles inside each assay unit to determine the sex ratio across treatments.

2.2.3. Effect of methyl jasmonate treatment on brood production of *Ips typographus*

Another experiment was conducted to investigate the effects of MJ treatment on brood production of *I. typographus*. We utilized two ramets of a different 40-year-old clone, which had been treated the same way as in Experiment 2. On 15 June 2004, the trees were felled and each ramet was cut into 40-cm bolts. This yielded 8 bolts where half the bark circumference was treated with MJ (MJ) and the other half was untreated (MJ_C), and 8 bolts where the whole bark area was untreated (Control). Pairs of bolts (one from each type) were placed in the forest in a randomized block design and baited with an Ipslure pheromone dispenser placed between the bolts. The distance between bolts within a pair was 50 cm and the distance between pairs was 50 m. Bolts were left in the field for 7 days for colonization by *I. typographus* and then brought to an out-door insectary. The bolts were split longitudinally through the center to separate the MJ and MJ_C treatments, and each half-bolt was sealed with melted paraffin wax on the cut surfaces to minimize desiccation. Control bolts were split and treated in the same way as the MJ-treated bolts. This procedure yielded 32 half-bolts that were hung individually inside fine mesh cloth bags with funnels; collecting bottles were attached to the lower end to collect emerging beetles.

In October-November 2004, we counted the number of beetles that had emerged from each half-bolt. Because there still were beetles in the bolts, we also estimated the number of remaining beetles by counting the numbers under two randomly chosen 100 cm^2 (10 × 10 cm) areas of bark on each bolt. Furthermore, we determined beetle dry weight by weighing 100

randomly selected beetles per bolt (dried at 110 °C for 60 minutes). Beetles were weighed to an accuracy of 0.01 mg.

2.2.4. Statistical analyses

Data were analyzed using analysis of variance. Each variable was tested to satisfy assumptions of normality and homogeneity of variances (Zar, 1996) by graphical analysis of residuals (Neter et al., 1983). If the variance was non-homogeneous, variables were transformed to square root, which provided distributions that satisfied these assumptions in all cases. Sex ratio and proportional data for *I. typographus* were transformed by arcsin$\sqrt{y}$. Beetle colonization data from experiment 1 were analyzed on a single-tree basis, using the calculated differences between MJ-treated and untreated bark within trees as the response variable. The data were subjected to one-sample t-tests using SYSTAT (SPSS, IL, USA). Dependent variables in experiments 2 and 3 were analyzed by repeated measure analysis in Proc Mixed (SAS Institute, 1996), as a split-plot, with randomized block design, treating sites, bark discs, or logs as blocks. For each variable, covariance parameter estimates for block and block x treatment were calculated in order to reveal whether there was any variability due to block or block by treatment interaction. In all experiments, blocks were accepted as a random factor, and if the covariance parameter of a block was equal to '0', the block term was eliminated from the random statement in the model. A Protected LSD test was used for multiple comparisons of means.

2.3. Effect of jasmonate treatment on defense responses to the white pine weevil (*Pissodes strobi*), and influence of mechanical wounding and weevil attack on terpene content

2.3.1. Plant material and treatment

The field experiments were conducted on a 15-year-old Norway spruce clonal bank located in the Valcartier research station, located 50 km north of Quebec City (46° 57' N-71° 28' O). These clones, initially selected for their cold hardiness and good growth

capacity under Quebec conditions, are currently being used in a breeding program. These Norway spruce trees were chosen because they can support the entire white pine weevil life cycle. One hundred and twenty 15-year-old Norway spruce trees chosen for the test had their terminal leaders protected with nylon cages. Treatments were randomly applied to these clones. For the field test, five different clones made up of four ramets were selected to monitor *TPS* gene expression. One treatment was assigned to each clone and samples were collected at the beginning of the experiment, then after 4, 12, and 32 d on a different ramet for each time point. Thus, three trees from the clone 84 462 were wounded using a scalpel by making eight 10-cm-long incisions along the stem, just above the first whorl at each cardinal point (two incisions for each point) (Figure 2.1B). Jasmonic acid (JA) treatments consisted of injecting either a 10 μM (clone 81 459) or a 100 μM (±) (clone 81457) JA solution directly into the stem, 10 cm below the first whorl (Figure 2.1A). This was performed by filling a 60-mL syringe with 35 mL of the JA solution and a 5-mm-diameter plastic tube (VWR, Mississauga, ON) was connected to the end of the syringe, inserted into a pre-drilled 1-cm-deep hole drilled into the stem, and allowed to drain into the tree's vascular system by gravity. The entire volume of the solution was absorbed within 6 h. Control trees (clone 84 416) were injected with 35 mL of water. A fifth treatment (clone 87 462) consisted of caging three mated white pine weevil females on the terminal leader (Figure 2.1C). For each time point, the entire terminal leader was cut off and flash-frozen in liquid nitrogen; the samples from needles and bark were then removed and kept separately in a plastic bag at -80ºC. In a preliminary test, an aqueous 5% blue food coloring preparation (McCormick, London, ON) was injected into a terminal leader to verify if gravity and the tree vascular system were capable of absorbing the entire volume of solution. Observation of a longitudinal section the terminal leader clearly demonstrated that the coloration was systemically and entirely distributed in the vascular tissue within less than 6 hours.

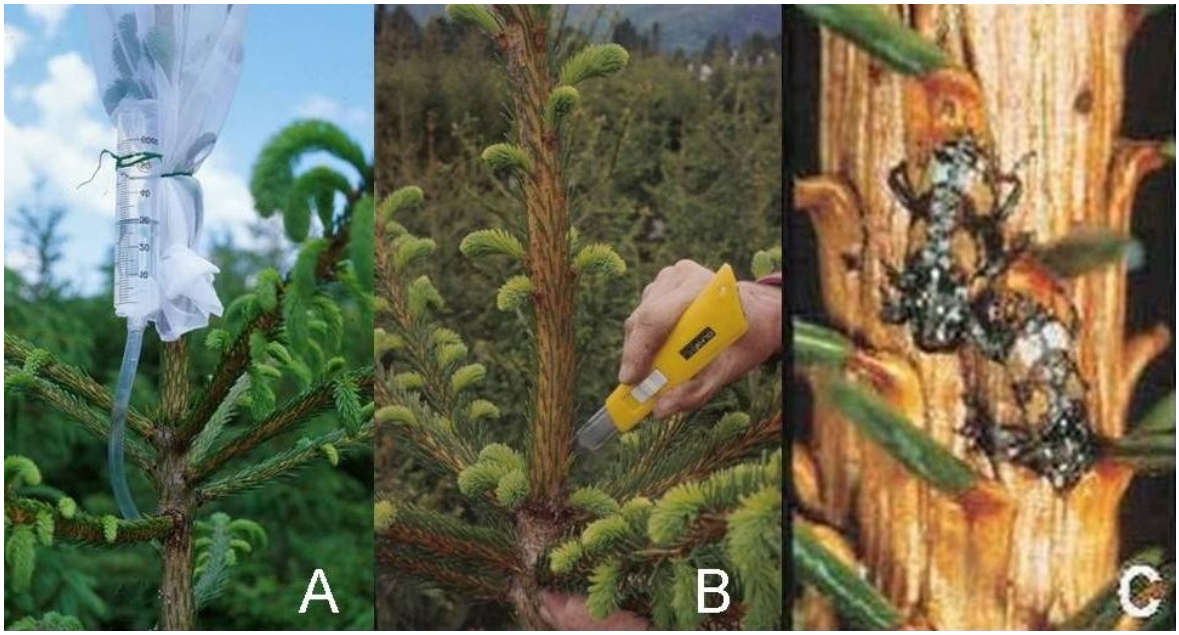

Figure 2.1. Illustration of treatments: (A) Injection of JA solution directly into the stem, 10 cm below the first whorl, (B) mechanical wounding of Norway spruce terminal leader, (C) Feeding activities of two white pine weevils on a Norway spruce terminal leader.

2.3.2. Preparation of attacked Norway spruce terminal leaders for histological examination

Histological analyses were performed on Norway spruce terminal leaders from the experimental material generated as previously described. Sixty days after treatment, a 1-cm transversal slice was cut 15 cm above the first whorl on 18 leaders (three samples from each treatment (jasmonic acid solution 10 µM and 100 µM, water injection, mechanical wounding, white pine weevil feeding, and one control). Sample disks were divided into four equal quadrants, put into vials, and covered with Tissue-Tek O.C.T. compound (CANEMCO Supplies, Saint Lawrence, QC) before being frozen at -20°C. Frozen samples were then cut (40 µm) with a HistoStat cryomicrotome (Reichert, Vienna, Austria). Sections were observed with a Polyvar light microscope (Reichert, Vienna, Austria) under blue light excitation using a BP 450-490 excitation filter combined with a DS 510 separator mirror and a LP 515 barrier filter. The blue illumination enabled detection of autofluorescent phenolic compounds and of resin ducts (Fernandez and Heath 1986,

Franceschi et al., 1998). Photographs were taken using Kodak Ektachrome Professional Film (Kodak, Guelph, Canada).

2.3.3. White pine weevil response to treatments

JA (10 μM and 100 μM solution), water (as a control), and wounding treatments were performed from 24 May 2001 to 31 May 2001 on 10 selected clones of Norway spruce. Each ramet of a clone received a different treatment. A control consisting of three mated white pine weevil females caged on the terminal leader was also used. After a 7-day period following treatments, three mated white pine weevil females were caged on each terminal leader, where they remained for the entire development of the next generation (Figure 2.1C). After 60 days, the shoots were cut and brought back to the greenhouse. Every two days, newly emerged adults were removed and weighed. At the end of the emergence period, feeding and oviposition holes and nymph chambers were counted and the data analyzed.

2.3.4. Statistical analysis

The experiment on the effects of *TPS*-like gene expression on the biological cycle of the white pine weevil was conducted using a split-plot design. The normality of the data was examined using PROC UNIVARIATE (SAS Institute, 1996). The numbers of feeding punctures, oviposition holes, and pupal chambers were normalized using a square root transformation. The statistical analysis, PROC MIXED procedures and orthogonal contrast were performed using the Statistical Analysis System (SAS Institute, 1996).

2.4. Extraction of resin terpenes

Terpene extractions were based on the procedures of Martin et al. (2002). All steps were carried out in 2-mL glass vials with teflon-coated screw caps (Hewlett-Packard, Palo Alto, CA). Sample pieces (200 mg) were submerged into 1.5 mL of *tert*-butyl methyl ether containing 150 μg mL^{-1} isobutylbenzene and 200 μg mL^{-1} dichlorodehydroabietic acid as internal standards, and extracted over 14 h with constant shaking at room temperature. To

purify extracted terpenes from other organic acids, the etheral extract was transferred to a fresh vial and washed with 0.3 mL of 0.1 M $(NH_4)_2CO_3$ (pH 8.0). The extract was then split into two equal portions. For the analysis of diterpene resin acids, one aliquot was methylated by adding 50 μL of 0.2 M N-trimethylsulfonium hydroxide in methanol (Macherey-Nagel, Dűrren, Germany) to 0.4 mL of the washed etheral extract in a separate vial. After incubation at room temperature for 2 hours, the solvent was evaporated under nitrogen to leave 100 μL of sample, which was stored at -20°C until analysis by GC-MS. For the analysis of monoterpenes and sesquiterpenes, the remainder of the original sample was prepared for GC-MS analysis by filtering through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous $MgSO_4$. The column was washed with 1 mL of diethyl ether, and the combined eluant collected in a fresh vial and evaporated to an approximate volume of 100 μL before storage at -20°C until analysis by GC-MS.

2.5. Analysis of monoterpenes, sesquiterpenes, and diterpenes

GC-MS analysis of monoterpenes and sesquiterpenes was carried out with a Hewlett-Packard 6890 GC-MS system, using a DB-WAX column (0.25 mm × 0.25 μm × 30 m, J&W Scientific, Folsom, CA). Split injections (1 μL etheral extract) were made at a ratio of 1:5 with an injector temperature of 220°C. The instrument was programed to hold an initial temperature of 40°C for 3 minutes and then increased at a rate of 3°C min^{-1} to 80°C. The temperature was then increased at a rate of 5°C min^{-1} to 180°C with a 5-minute hold, followed by a final ramp of 15°C min^{-1} up to 240°C. Helium was used at a constant flow of 1 mL min^{-1}. For the identification of compounds, the MS detector was operated using a mass range of 40-350 for monoterpenes and sesquiterpenes and a range of 40-550 for diterpenes, and spectra were collected under standard conditions (electron impact ionization at 70 eV). Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards or to mass spectra in the Wiley or National Institute of Standards and Technology libraries.

Analysis of diterpene constituents was performed on the same GC-MS instrument fitted with an HP-5 column (0.25 mm × 0.25 μm × 30 m, Hewlett-Packard). Injections

were made with 1 μL of the concentrated, derivatized etheral extract. GC-MS split ratios were 1:10 with an injector temperature of 220°C. The instrument was programed for an initial temperature of 120°C and increased at a rate of 1°C min^{-1} to 150°C, followed by 5°C min^{-1} up to 280°C (6 minutes hold). Helium was used as carrier gas at a constant flow of 1 mL min^{-1}.

Table 2.1. Authentic standards and their origin

NO.	COMPOUND IDENTIFIED AS:	STANDARD	ORIGIN
1	tricyclene	tricyclene	Fluka (Buchs, Switzerland)
2	α-pinene	(+)-α-pinene and (-)-α-pinene	Fluka (Buchs, Switzerland)
3	camphene	(-)- campene	Fluka (Buchs, Switzerland)
4	sabinene	Sabinene	Roth (Karlsruhe, Germany)
5	β-pinene	(+)-β-pinene and (-)-β-pinene	Fluka (Buchs, Switzerland)
6	myrcene	myrcene	Fluka (Buchs, Switzerland)
7	3-carene	(+)-3-carene	Fluka (Buchs, Switzerland)
8	β-phellandrene	(R)(-)-β-phellandrene	Fluka (Buchs, Switzerland)
9	limonene	(R)(+)-limonene and (S)(-)-limonene	Fluka (Buchs, Switzerland)
11	para-cymene	para-cymene	Aldrich (Steinheim,Germany)
12	β-ocimene	β-ocimene	Fluka (Buchs, Switzerland)
13	α-terpinolene	α-terpinolene	Fluka (Buchs, Switzerland)
14	bornyl acetate	(-) bornyl acetate	Sigma (Steinheim, Germany)
15	α-longipinene	(+)- α-longipinene	Fluka (Buchs, Switzerland)
16	longifolene	(+) Longifolene	Fluka (Buchs, Switzerland)
17	(*E*)-β-caryophyllene	(*E*)-(-)-β-caryophyllene	Fluka (Buchs, Switzerland)
18	α-humulene	(-)-α-humulene	Fluka (Buchs, Switzerland)

19	germacrene D	*Alosya sellowii* Oil	(T. Koellner, MPI Jena)
20	δ-cadinene	*Alosya sellowii* Oil	(T. Koellner, MPI Jena)
21	β-farnesesne	β-farnesesne	Bedoukian (Danbury CT. USA)
22	isobutyl benzene	isobutyl benzene	Across organics (NJ-USA)
23	pimaric acid	pimaric acid	Helix Biotech (BC, Canada)
24	sandaracopimaric acid	sandaracopimaric acid	Helix Biotech (BC, Canada)
25	isopimaric acid	isopimaric acid	Helix Biotech (BC, Canada)
26	levopimaric acid	levopimaric acid	Helix Biotech (BC, Canada)
27	dehydroabietic acid	dehydroabietic acid	Helix Biotech (BC, Canada)
28	abietic acid	abietic acid	Fluka (Buchs, Switzerland)
29	neoabietic acid	neoabietic acid	Helix Biotech (BC, Canada)
30	dichlorodehydroabietic acid	dichlorodehydroabietic acid	Helix Biotech (BC, Canada)

GC-MS generated peaks were quantified using Hewlett-Packard Chemstation software. Isobutylbenzene was used as the internal standard for quantification of both monoterpenes and sesquiterpenes while the methylated dichlorodehydroabietate was employed as an internal standard to calculate diterpene concentrations. For quantitative analysis of monoterpenes and sesquiterpenes, the MS detector was operated in the single ion mode monitoring ions at 91, 93, and 161 *m/z*, corresponding to the internal standard, monoterpenes, and sesquiterpenes, respectively. (Quantitation of the sesquiterpenes β-caryophyllene and α-humulene also relied on the 93 *m/z* ion). For verification, the [M^+] ions of the internal standard, monoterpenes, and sesquiterpenes (corresponding to 134, 136, and 204 *m/z*, respectively) were also monitored. For diterpene quantification, the following selected ions were monitored: 121 *m/*z (methyl sandaracopimarate), 135 *m/*z (methyl abietate), 239 *m/z* (methyl dehydroabietate), 241 *m/z* (methyl pimarate and levopimarate), and 307 *m/z* (methyl dichlorodehydroabietate). The following ions were monitored for verification during diterpene analysis: 314 *m/z* (methyl dehydroabietate), 316 *m/z* (methyl pimarate, sandaracopimarate, levopimarate, and abietate), and 382 *m/z*

(methyl dichloroabietate). The total monoterpene, sesquiterpene, or diterpene resin acid content was calculated as the sum of the individually quantified compounds.

The enantiomeric ratios of the major monoterpene hydrocarbons were determined using a FS-Hydrodex, Heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin/OV-1701 capillary column (30m, 0.25mm ID, 0.25 μm film thicknes, Macherey-Nagel, Dűrren, Germany). The instrument was programed for an initial temperature of 45°C (10-minute hold) and ramp of 1°C min^{-1} to 80°C, followed by a second ramp at 5°C min^{-1} until 160°C (5-minute hold). Identification of enantiomers was performed by comparing GC retention times to those of enantiomerically pure standards purchased from Sigma-Aldrich (Steinheim, Germany).

2.6. Extraction and identification of phenolic compounds

Extraction of phenolic constituents was modified from Laitinen et al. (2002). The plant material was weighed (100 mg) and extracted with 2.5 mL of methanol (100%) using an Ultra-Turrax homogenizer for 30 s, after which the samples were left for 15 minutes on ice. The samples were then centrifuged (16000*g*, 3 minutes) and decanted. The remaining pellet was extracted three more times by being homogenized for 30 s, placed for 2 minutes on ice, and then centrifuged. The combined supernatants were vacuum-evaporated to dryness, re-dissolved in 1 mL of 100% methanol, and transferred to a fresh vial for analysis. After being evaporated to dryness again under nitrogen, the extracts were stored at -20°C.

The quantitative analysis of phenolics of wood and bark samples was carried out by high-performance liquid chromatography (HPLC) using a Hewlett-Packard system with a quaternary pump (HP 1050), an autosampler (HP 1100), a photodiode array detector (HP 1100), HP ChemStation Software, and a 3 μm Hypersil OSD column (60 x 4.56 mm ID) (Phenomenex, Lyna). The mobile phases used were 0.2% trifluoroacetic acid (A) and acetonitrile (B). The samples were redissolved in 0.4 mL 50% methanol and separated according to the following gradient: 10 to 50% B (30 minutes), 50 to 100% B (2 minutes), followed by a return to initial conditions over 7 minutes. The flow rate was 0.8 mL/min and the injection volume 10 μl. Spectra were recorded from 230 nm to 400 nm (range step

2.00 nm) with a threshold of 1.00 mAU, while the quantification was done at 230 nm. Compounds were identified based on comparisons of the retention times and the spectral characteristics with authentic standards purchased from Extrasynthèse (Genay, France; apigenin and apigenin 7-O-glucoside), Polyphenol AS (Sandnes, Norway; piceatannol and piceid), Sigma-Aldrich (Steinheim, Germany; catechin hydrate and taxifolin), or ICN Pharmaceuticals (Costa Mesa, CA; kaempferol and resveratrol). Analytes were quantified using calibration curves prepared with authentic standards, and response factors were based on quercetin (Roth, Karlsruhe, Germany). In all wood samples an unidentified compound was found with a molecular mass of 714 whose UV spectrum and HPLC retention time suggested a polar phenolic compound. Its abundance was not affected by methyl jasmonate treatment.

2.7. The culture of plant cells

2.7.1. Cell line initiation and maintenance

Embryogenic tissue (cell line *Pa. 186.3*) was initiated from mature zygotic embryos. Until embryo isolation, the seeds were stored at –20°C. After isolation, the embryos were laid flat on the culture medium and cultured in darkness at 24°C for 4–10 weeks until embryogenic tissue had emerged and could be isolated. During this period, embryos were transferred to fresh medium every third week (see Walter et al., 1999). The pieces of embryogenic tissue were suspended in BMIS1 medium at the Norwegian Institute of Forest Research (Ås, Norway) and about 0.5 gram of the culture was transferred to cryo vials (Nunc) and sent to Germany by express mail.

For long-term storage, callus was kept in cryopreservation. Tissue was maintained in Embryo Germination Medium (EGM) (Smith, 1996) supplemented with DMSO and sorbitol. Every six months, callus was transferred to plates supplemented with EDM6 medium plus Gelrite (0.32%) and subcultured fortnightly.

Suspensions were initiated from callus by inoculating 40 mL of EDM6 medium in 125-mL Erlenmeyer flasks with ca. 0.5 g of callus. Suspensions were typically subcultured every 10 days by adding 4 mL of conditioned medium and cells to 36 mL of fresh EDM-6

medium in 125-mL Erlenmeyer flasks (Schott, Jena, Germany). The embryogenic tissue lines were maintained on modified "Embryo Development Medium" (EDM6). The medium used for both maintaining cell lines and experiments was EDM-6 (Smith, 1996; Walter et al., 1999) and consisted of macro-salts (KNO_3, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $NaNO_3$, and $NH_4H_2PO_4$), micro-salts ($MnSO_4.4H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, KI, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, and $CoCl_2.6H_2O$), monocarbohydrates ($FeSO_4.7H_2O$, and Na_2EDTA), and vitamins (Thiamine HCl, Nicotinic acid, and Pyridoxine HCl). The pH was adjusted to 5.8±0.1 prior to autoclaving. Filter-sterilized amino acids (Arginine, Glutamine, Citruline, Ornithine, Lysine, Alanine, and Proline), were added into cooled medium supplemented with 5μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.5 μM N^6-benzyladenine (BA). Cultures were kept in Innova^{TM} refrigerated incubator shakers Model 4230 (New Brunswick Scientific, Edison, NJ). All tissue cultures were maintained at 24 ± 1°C in darkness.

2.7.2. Culture elicitation and metabolic studies measurements

Liquid cultures were started from established callus and subcultured at ten-day intervals. Cultured cells were assayed for both endogenous monoterpene production and monoterpene synthase activity using two elicitors, 50 μM methyl jasmonate (MJ) (Sigma-Aldrich, Steinheim, Germany) or 100 ug/mL chitosan from crab shells (Sigma-Aldrich, Steinheim, Germany). Chitosan was prepared based on the protocol of Walker-Simmons et al. (1984). Briefly, 100 mg of chitosan was dispersed in 9.5 mL of water, and 30 μL glacial acetic acid was added to fully dissolve the sample. The pH was adjusted to 7.0 with 1 N NaOH and the volume was adjusted to 10 mL with water. All samples were heated in a boiling water bath for 20 minutes to ensure sterility before testing.

To sample cultures for endogenous monoterpene production, *Picea abies* cell line *P.a. 186.3* was grown for 3 days in EDM6 suspension medium. Suspension cultures were elicited with either 50 μM methyl jasmonate or 100 ug/mL chitosan final concentration. Sterilized XAD-4 resin (Sigma-Aldrich, Steinheim, Germany) was added to a control or induced culture 1 hour after the addition of elicitors, along with isobutylbenzene (1 μg/mL final concentration) as internal standard, and grown for another 7 days. Control cells were

grown for the same period of time without adding elicitors. Ten independent samples were assayed for each treatment and control. On day 10, the XAD-4 resin and cells were then harvested by filtering them onto Whatman paper to remove the medium, using a Büchner funnel with vacuum. Cells and resin were transferred to 30 mL Pyrex Culture Teflon-lined screw-capped tubes (Sigma-Aldrich, Steinheim, Germany). After adding 15 mL of pentane, the tubes were agitated vigorously for at least 4 hours at room temperature. The extract was purified by filtering through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous $MgSO_4$ and collected into clean glass vials. The purified extract was concentrated on ice under a gentle nitrogen steam to about 200 µL and then analyzed by GC-MS as described above (see 2.5).

To monitor the effect of treatment on cell growth, 3-day-old cultures were treated with either 50 µM methyl jasmonate (MJ) or 100 ug/mL chitosan or water and harvested at time of inductions, 5, 7, 9, and 10 days post-induction. All time points were assayed in triplicate. Cells were harvested by filtering them onto Whatman paper to remove the medium, using a Büchner funnel with vacuum. Growth of the cells was determined gravimetrically. The filtered medium was used to monitor changes in the medium pH and the effect of treatments on the pH and to correlate with cell growth.

To monitor terpene synthase activities, 7 day-old cultures were treated with either 50 µM methyl jasmonate (MJ) or 100 ug/mL chitosan or water and harvested at time of inductions, 20 minutes, 40 minutes, and 1, 2, 4, 8, 12, 24, 48, 72, or 96 hours post-induction. All time points were assayed in triplicate or quadruplicate. Harvested cells were filtered and stored at −80 °C prior to extraction. Monoterpene synthase activity was determined by published procedures (Lewinsohn et al., 1993; Bohlmann et al., 1997; Martin et al., 2002) with minor modifications. Before assaying enzyme activity, the frozen protein extracts were placed at 37°C until just thawed. The protein extracts were desalted in Bio-Rad Econo *Pac*I0DG sizing columns pre-equilibrated with appropriate assay buffers: mono-TPS buffer (25 mM HEPES, pH 7.5; 5 mM DTT; 10% [v/v] glycerol; 1 mM $MnCl_2$; and 100 mM KCl), sesqui-TPS buffer (25 mM HEPES, pH 7.3; 10 mM $MgCl_2$; 10 mM DTT; and 10% [v/v] glycerol), or di-TPS buffer (30 mM HEPES, pH 7.2; 7.5 mM $MgCl_2$; 20 µM $MnCl_2$; 5% [v/v] glycerol; and 5 mM DTT). Enzyme activity was assessed with 1 mL of the desalted extracts with the addition of 10 µM GPP (with 1 µCi ^{3}H-GPP)

(Biotrend, Köln, Germany) for mono-TPS activities, or 10 μM GGPP (0.5 μCi ^{3}H-GGPP) (Biotrend, Köln, Germany) as substrate for di-TPS assays. All enzyme assays were done in triplicate, overlaid with 1 mL of pentane to collect released volatiles, and incubated at 30°C for 1 hour. To stop all enzyme activity, the extracts were immediately placed on ice. The aqueous assay fraction was rapidly extracted with the pentane fraction by vortexing, and separation of the aqueous and organic fractions was achieved by centrifugation at 12,500*g* for 2 minutes. A 0.9-mL aliquot of the original 1.0 mL pentane overlay was removed and filtered through a Pasteur pipette filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.4 g of $MgSO_4$ to remove nonspecific substrate hydrolysis products and to dry the pentane extract. Each enzyme assay was extracted with an additional portion of pentane, vortexed, and centrifuged as before. These sequential extractions were also passed over the same column and pooled with the initial column eluent. Subsequently, the column was washed with 1 mL of pentane and the total volume was determined. The extracts were analyzed by liquid scintillation counting, 0.8 mL in 4 mL of Lipoluma (J.T. Baker, Deventer, The Netherlands). The conditions for all enzyme assays, including pH optimum, incubation time, substrate concentration, and temperature optimum were optimized for this system so that maximum activity was achieved in a linear range of product generation.

RESULTS

3.1. Methyl jasmonate, terpenes and resistance to *Ceratocystis polonica*

3.1.1. Terpene profile of untreated *Picea abies* wood and bark tissues

To quantify the effects of methyl jasmonate treatment on the resin of Norway spruce, the constitutive terpene levels and composition in the sapwood and the bark tissue of untreated trees was measured. In the present study, twenty-eight compounds were detected and identified by means of GC-MS analyses. Monoterpenoids are the most abundant, represented by fourteen compounds, the majority of them (twelve) monoterpene hydrocarbons, while sesquiterpenoids were represented by six sesquiterpene hydrocarbons. The most abundant constitutive monoterpenes were α-pinene and β-pinene followed by limonene. A representative GC-MS chromatogram is shown in Figure 3.1, while the chemical structures of the major mono- and sesquiterpenes are shown in Figure 3.2.

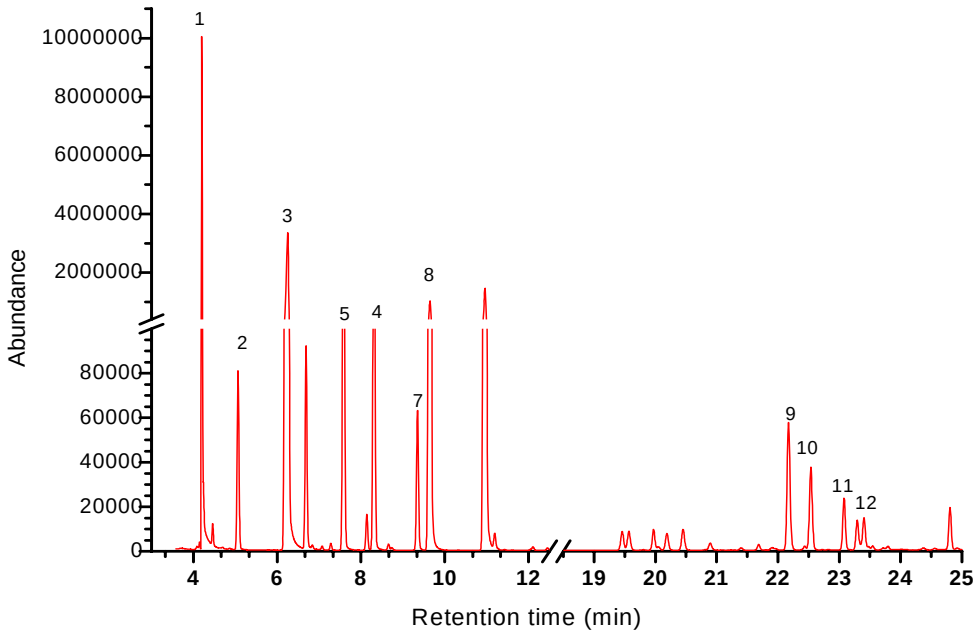

Figure 3.1. GC-MS chromatogram of etheral extract of 40-year-old Norway spruce trees showing the presence of terpenoid compounds.

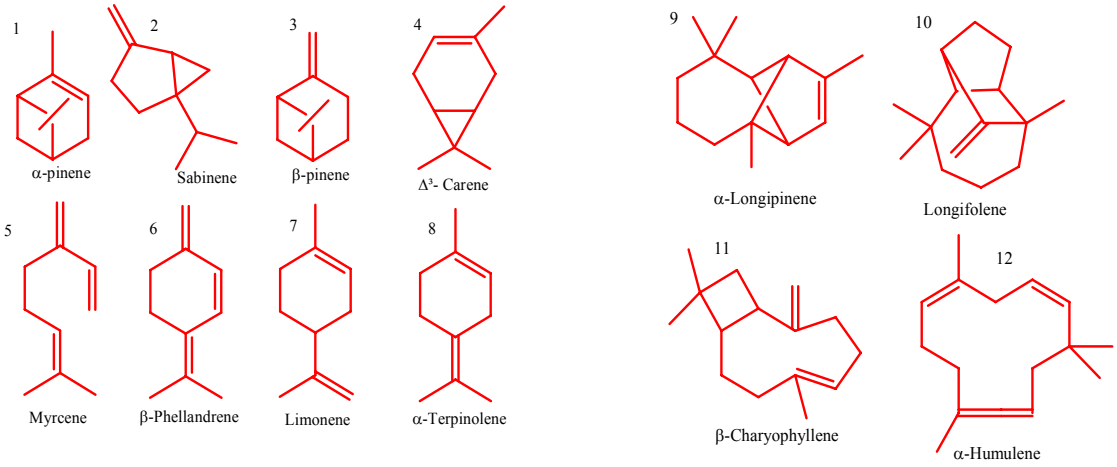

Figure 3.2. The chemical structures of the major monoterpene (1-8) and sesquiterpene (9-12) compounds found in the spruce wood and bark tissues.

Diterpenes in spruce resins were characterized by two main skeletal types: pimarane (Figure 3.3A) and abietane (Figure 3.3B); each of the types represented by four compounds. The same diterpene resin acids were found in both wood and bark tissue. Labdane-type acids, which may contain conjugated diene compounds that readily polymerize, were not found.

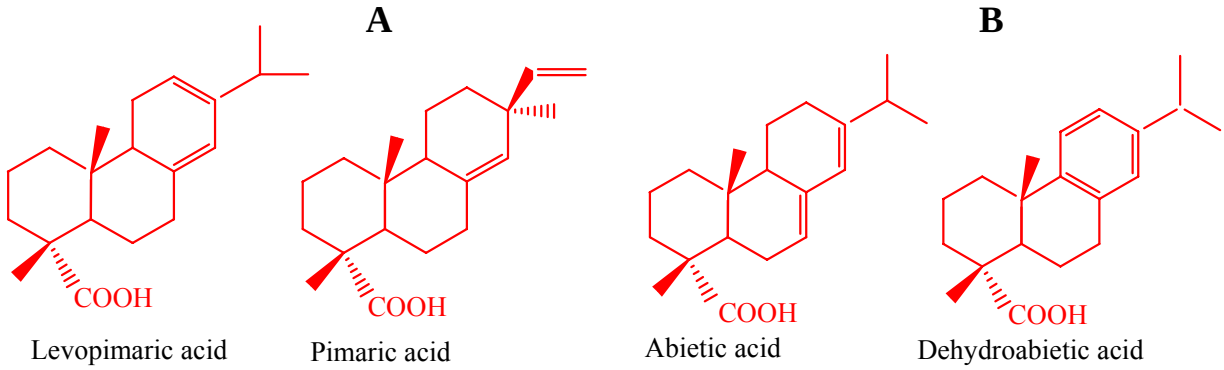

Figure 3.3. The chemical structures of the major diterpene compounds found in spruce wood and bark tissues.

3.1.2. Methyl jasmonate increases resin ducts and resin flow

To quantify the induced resin response of Norway spruce after methyl jasmonate treatment, traumatic resin duct formation and terpene accumulation in the sapwood, and external resin flow on the bark surface was measured. In control trees, axial resin ducts were

largely restricted to the bark (all tissues outside of the cambium, i.e. phloem, cortex, and periderm). However, after MeJA treatment, striking morphological changes became apparent. Examination of the anatomy of the treated trees in the multiple clone experiment revealed that methyl jasmonate had stimulated the formation of a ring of new resin ducts (traumatic resin ducts) in the newly formed xylem (Figure 3.4B). As seen in Fig. 3.4B, some new xylem cells immediately adjacent to the cambium had denser cytoplasm and thinner walls than surrounding xylem cells and appear to constitute the epithelial cells of nascent TDs which surround the terpene-rich lumen (Fig. 3.4B).

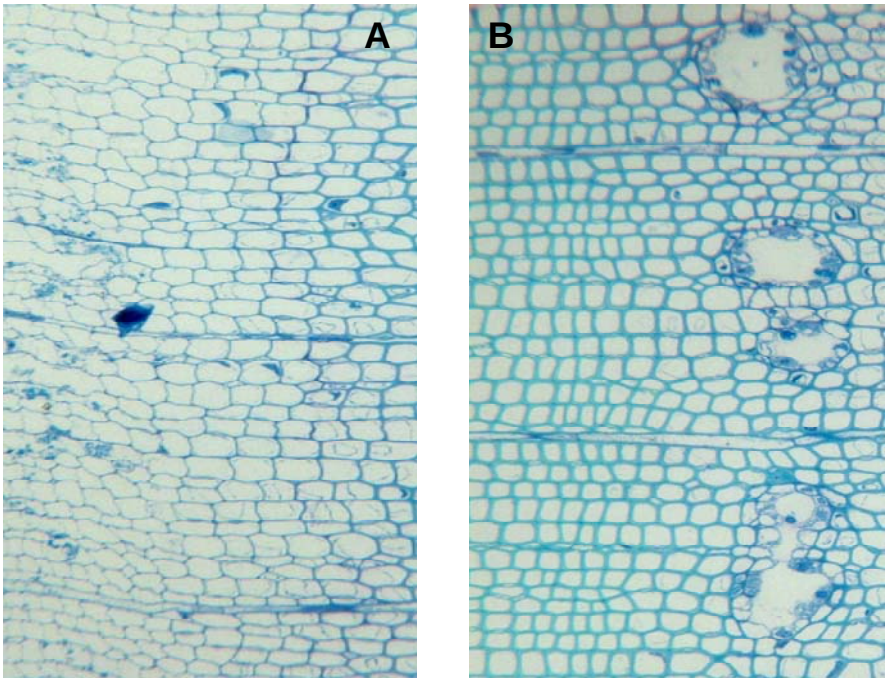

Figure 3.4. Light microscopy of induced traumatic duct (TD) differentiation in cross-sections of Norway spruce stems. (A) Untreated controls show the absence of resin ducts in the constitutive xylem and phloem. (B) Thirty days after 100 mM methyl jasmonate (MJ) treatment, accumulation of resin in the lumen of the fully differentiated TD visualized by staining with copper acetate is shown.

A dose-dependent response was observed in which trees treated with the highest concentration of methyl jasmonate demonstrated massive increases in traumatic resin duct formation and external resin flow compared to trees from the control group, but generally lower doses did not cause significant changes (Figure 3.5).

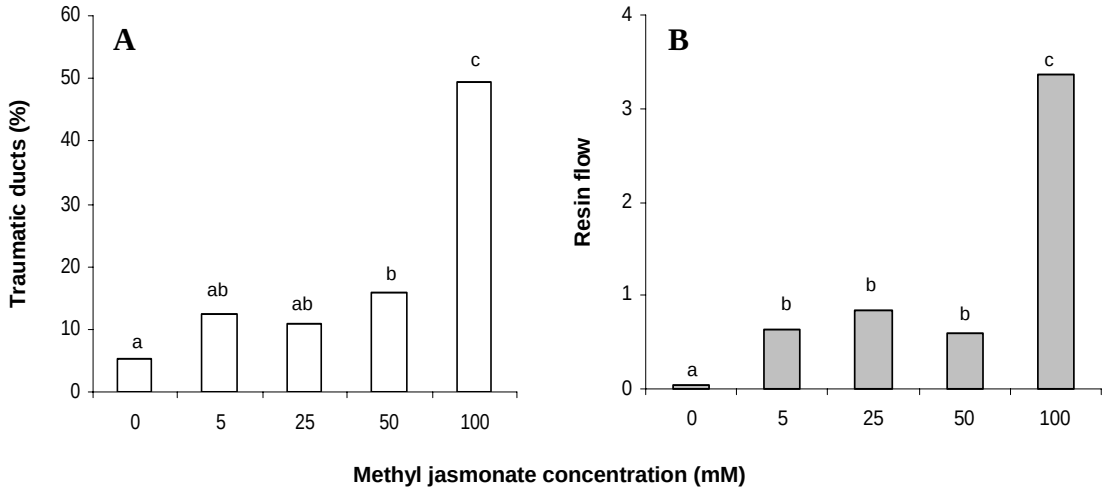

Figure 3.5. Effect of various methyl jasmonate treatments on traumatic resin duct formation (A) and bark resin flow (B). Values shown are averages of all clones. Traumatic ducts are expressed as the percentage of coverage in sample sections in a region 1.1 mm across in the tangential direction four weeks after methyl jasmonate treatment. Resin flow was quantified four weeks later. Bars with the same letter were not significantly different (LSD test a P=0.05 following ANOVA).

Trees treated with 100 mM methyl jasmonate produced more than 25 times as many traumatic ducts as controls, and the external resin flow on the trunk was more than 80 times higher (Figures 3.5 and 3.6). There was a strong, positive correlation between these parameters ($p < 0.0001$, $R^2 = 0.80$).

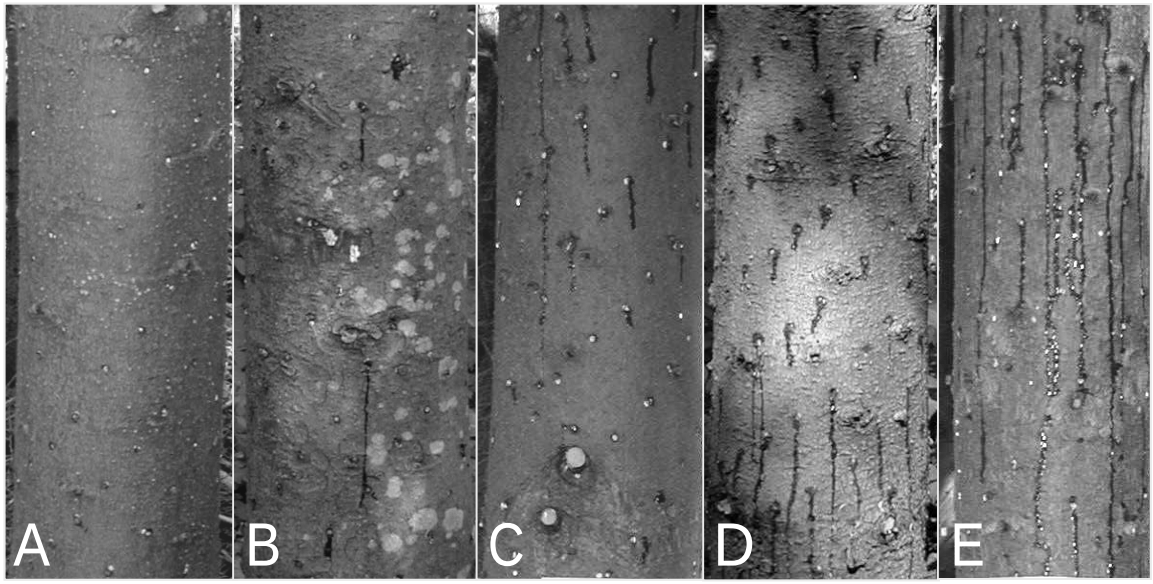

Figure 3.6. Representative outer bark surfaces of *P. abies* treated with different concentrations of methyl jasmonate or left as a control. The sections depicted correspond to values of 0 (A), 1 (B), 3 (C), 4 (D), and 5 (E) on the relative scale used. Data on the effect of treatment on resin flow are given in Figure 3.5B.

3.1.3. Methyl jasmonate increases terpene content of *Picea abies*

The total content of monoterpenes plus sesquiterpenes in 100 mM-treated trees was 2.2 times higher than that of untreated controls (Figure 3.7), but there were no significant differences between other treatments ($F_{4,29}$ = 2.19, p = 0.11). However, trees that survived mass-inoculation with *C. polonica* had accumulated much higher monoterpene and sesquiterpene concentrations in response to methyl jasmonate treatment than trees that were killed by the fungus. The increases in total monoterpene and sesquiterpene concentration during the first four weeks after methyl jasmonate treatment was 3.47 mg g^{-1} fresh tissue in surviving trees, compared with 0.36 mg g^{-1} in trees that eventually were killed (p = 0.05, F = 4.36). Treatment with methyl jasmonate had no apparent effect on the polyphenolic parenchyma (PP) cells. Although the three clones that were analyzed for such cells (76, 80, 85) generally responded strongly to methyl jasmonate treatment, there were no significant differences between treatments in percentage of coverage of PP cells ($F_{4,29}$ = 1.35, p = 0.26) or the size of stained bodies within them ($F_{4,29}$ = 1.67, p = 0.16).

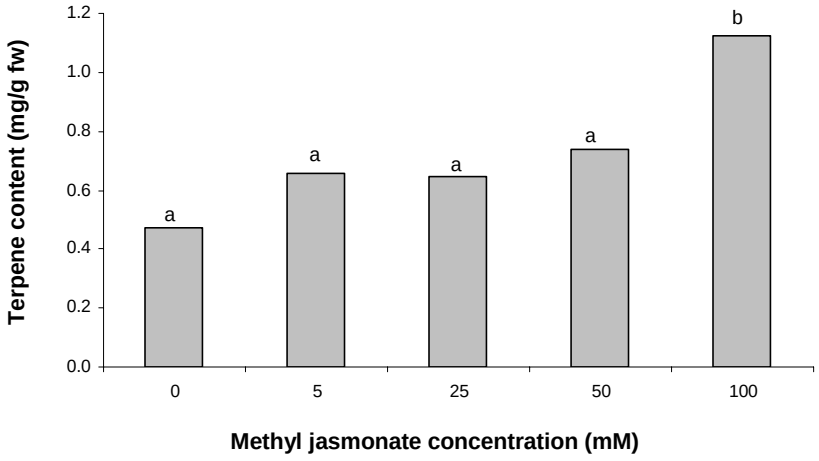

Figure 3.7. Effect of various methyl jasmonate treatments on terpene content. Values shown are averages of all clones. Terpene content is presented as mg g^{-1} fresh weight measured four weeks after

methyl jasmonate treatment. Bars with the same letter were not significantly different (LSD test a *P*=0.05 following ANOVA).

3.1.4. Clonal variation in resin response to methyl jasmonate treatment

Significant variation in resin response was observed among the clones used in this study (Figure 3.8). At one extreme, trees of clone 63 showed little response to methyl jasmonate treatment; there was no evidence of traumatic resin ducts, no changes in monoterpene and sesquiterpene content, and fungal growth was not reduced compared to the untreated control. At the other extreme, clones 76 and 80 showed the strongest induction of traumatic ducts and monoterpene and sesquiterpene levels, with a 3-4 fold increase in terpene content one month after treatment. Clone 126 showed a similar pattern of response as clones 76 and 80, but the increases in monoterpene and sesquiterpene content were not as dramatic. The other two clones had different response patterns. Clone 85 showed a 2-3 fold increase in monoterpene and sesquiterpene content at a methyl jasmonate concentration of 5 mM, but there was no significant change in terpene content at higher methyl jasmonate concentrations. The increase in traumatic ducts and the decline in fungal growth were also much more evident at 5 mM methyl jasmonate in this clone than in the other clones. Clone 75 showed little change in terpene content one month after methyl jasmonate treatment.

Terpene composition was only slightly affected by methyl jasmonate treatment and only in certain clonal lines. All clones contained the same basic complement of monoterpenes and sesquiterpenes, but differed in the relative amounts of some of these substances. The most abundant constitutive monoterpenes in the stems of all clones were α-pinene and β-pinene. In clones 63, 75, 76, and 80, β-pinene predominated over α-pinene, while in clones 85 and 126 the reverse was true. Clones 85 and 126 were the only ones that showed slight compositional changes over the sampling period, but the methyl jasmonate treatment had no effect. For example, in clone 126 trees in the first and second samplings had higher amounts of α-pinene (40-43 %) compared to β-pinene (24 – 28 %), but in the third sampling higher amounts of β-pinene were observed (42 – 47 % compared to 26 – 29 % α-pinene).

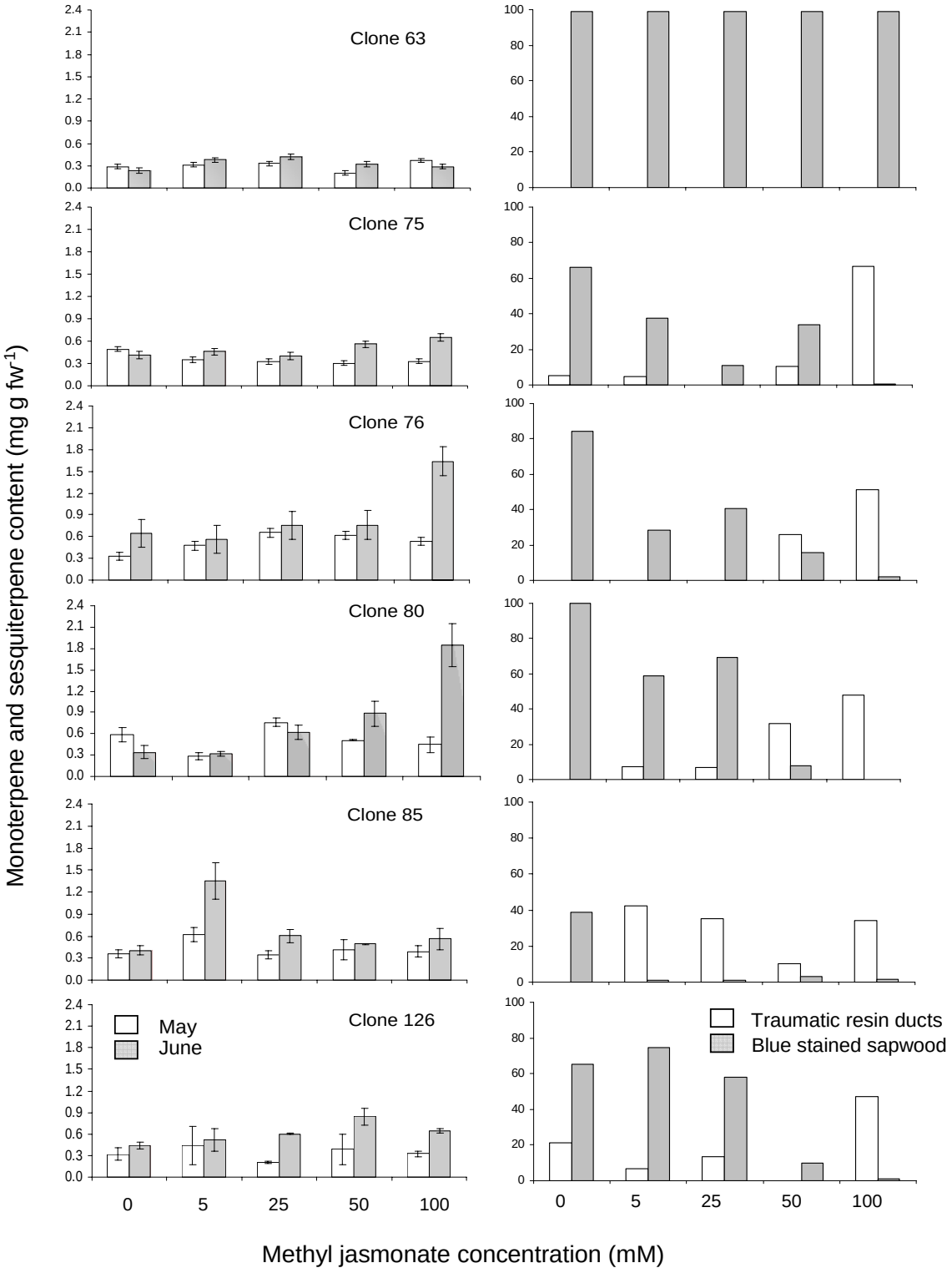

Figure 3.8. Effect of methyl jasmonate treatment on terpene content (left panels) and traumatic resin duct formation (unshaded bars, right panels) and sapwood blue-staining (shaded bars, right panels) of individual *P. abies* clones. Terpene response was measured at the end of May (just prior to methyl

jasmonate application), and at the end of June (four weeks later, at the time of fungal inoculation). Traumatic resin ducts and blue-stained sapwood were measured only in samples taken at the end of June.

The most variable monoterpene was Δ-3-carene, the content of which varied from only 0.5-1% of the total monoterpene content in clones 63 and 85 to relatively high levels (14 – 30 %) in clones 75 and 80 in September samples, composing up to 44 % of the total monoterpene content. Gas chromatographic analysis using an enantioselective column indicated several monoterpenes were present in both enantiomeric forms. (-)-β-Pinene and (-)-limonene always predominated over their corresponding antipodes, comprising 90-96 % and 58-62 %, respectively, of the enantiomeric mixture in each case. For α-pinene, the (+) enantiomer was most abundant, representing 67-73 % of the total α-pinene content. Stereochemical variation among trees and treatments was low for all compounds considered in this study.

3.1.5. Methyl jasmonate increased monoterpenes, sesquiterpenes, and diterpenes, but not phenolics

To get a clearer picture of the chemical responses to methyl jasmonate treatment, we analyzed both terpenoids and phenolics in a single clonal line treated with 100 mM methyl jasmonate. Of the major classes of compounds, both monoterpenes/sesquiterpenes and diterpenes increased about 2-fold in methyl jasmonate-treated trees 35 days after treatment as compared to control trees (t = 5.56, P = 0.031) (Figure 3.9). An increase in the number of traumatic resin ducts was observed following methyl jasmonate treatment, from zero at the time of treatment, to nine at days 8 and 35. No traumatic resin ducts were observed in the control trees over the same period.

The main monoterpenes present were α-pinene, β-pinene, limonene, 3-carene, and β-phellandrene, which made up 82% of the total terpene fraction (Table 3.1). The sesquiterpene fraction was found to consist mainly of longifolene and δ-cadinene, and the diterpene fraction of levopimaric acid, dehydroabietic acid, and 5 other major compounds. However, there were few changes in terpene composition following methyl jasmonate treatment, with β-pinene showing an increase from 16 to 21 % at 35 days after methyl jasmonate treatment at the

expense of α-pinene (57 to 48 %). There were no observed changes in the enantiomeric composition of any chiral terpenes.

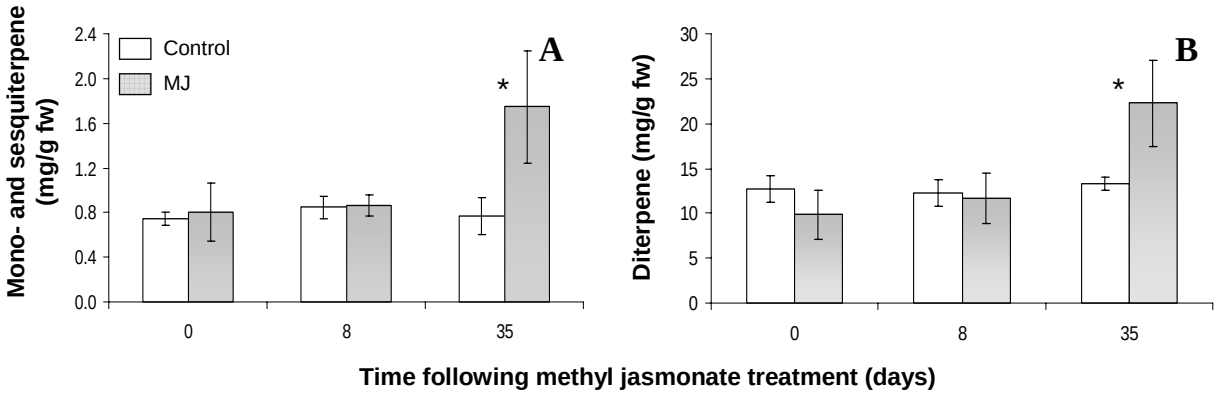

Figure 3.9. Effect of 100 mM methyl jasmonate treatment on (A) monoterpenes/ sesquiterpenes and (B) diterpenes content of a single clonal line (Clone 123). The "*" indicates significant differences between treatment and control.

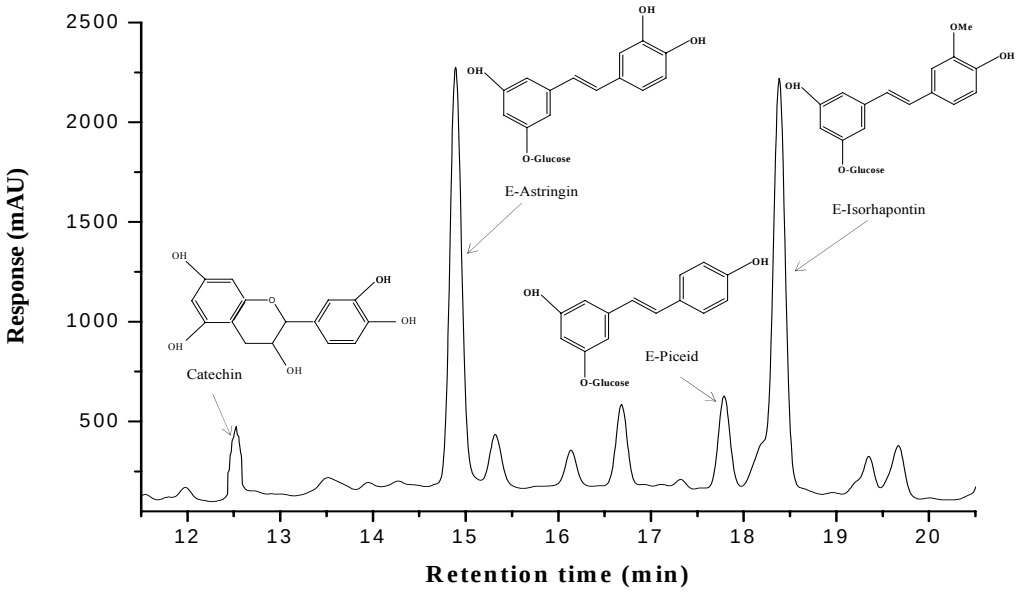

Figure 3.10. HPLC chromatogram of methanol extract of Norway spruce trees showing the presence of soluble phenolic compounds. The chemical structures of the major compounds are shown.

We looked for changes in the soluble phenolic content of *Picea abies* mature trees after methyl jasmonate spraying by HPLC analysis of methanol extracts of the bark (Figure 3.10). There were many more soluble phenolics in the bark than in the wood, both in terms of number of individual compounds detected and total quantities (Table 3.1), but only six major phenolic compounds were identified in bark and wood samples. The main constitutive phenolics present in the bark and wood of *Picea* species were stilbene glycosides, including astringin (3, 3', 4', 5-tetrahydroxystilbene 3-O-β-D-glucoside) and isorhapontin (3, 4', 5-trihydroxy-3'-methoxystilbene 3-O-β-D-glucoside). These stilbene glycosides comprised more than 85% of the total soluble phenolics found in both xylem and bark, with smaller amounts of catechin and the stilbene glycosides piceatannol and piceid.

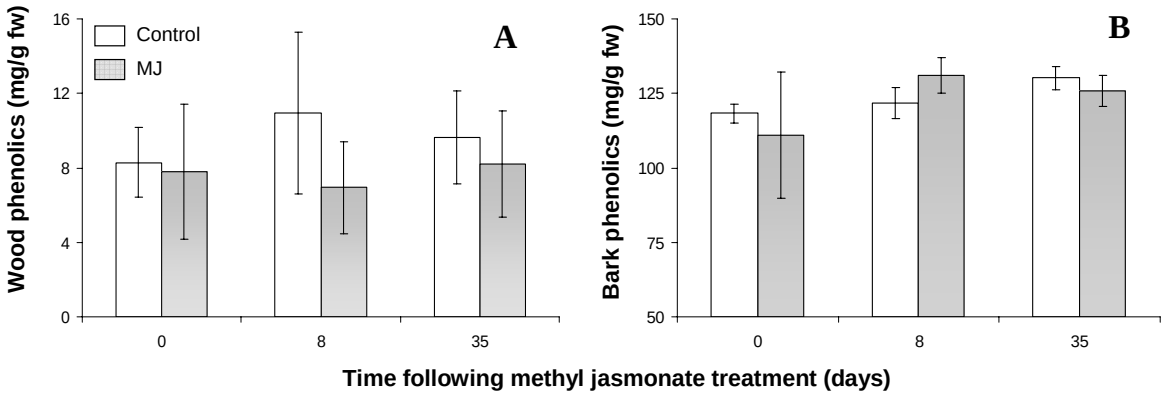

Figure 3.11. Effect of 100 mM methyl jasmonate treatment on phenolic content of (A) wood and (B) bark tissues of a single clonal line (Clone 123).

Although the PP cells showed anatomical changes similar to those found after fungal infection, there were no quantitative differences in phenolic content between MJ-treated trees (bark or wood) and untreated trees, neither for major stilbenes or flavonoid compounds ($P >$ 0.18, one-sample t-test) nor for total soluble phenolics over a period of 4 weeks after treatment (Figure 3.11). No significant differences were observed 8 days after treatment, and there were no changes in phenolic compounds in either bark or wood at any time point.

Table 3.1. Effect of 100 mM methyl jasmonate treatment on composition of *P. abies* terpenes and phenolics at different times following application. Lines in bold face show compounds that underwent the biggest changes and the total.

CHEMICAL CLASS	% COMPOSITION OF EACH CHEMICAL CLASS					
	Control			Methyl jasmonate treatment		
Monoterpenes and Sesquiterpenes	0 days	8 days	35 days	0 days	8 days	35 days
Tricyclene	0.2	0.2	0.2	0.2	0.2	0.2
α-Pinene	**57.5**	**53.8**	**57.4**	**57.6**	**55.4**	**48.4**
Camphene	0.6	0.6	0.6	0.6	0.6	0.5
β-Pinene	**15.9**	**17.1**	**15.9**	**16.3**	**16.4**	**21.8**
Myrcene	1.1	1.9	1.1	1.4	1.1	1.5
Δ-3-Carene	2.4	2.5	2.8	1.7	2.9	1.4
β-Phellandrene	4.7	5.8	4.6	4.6	4.9	6.1
Limonene	**12.6**	**13.3**	**12.5**	**12.7**	**13.2**	**16.3**
α-Terpinolene	0.1	0.1	0.1	0.1	0.0	0.1
Longifolene	3.3	2.9	2.8	2.9	3.2	2.2
(*E*)-β-Caryophyllene	0.4	0.5	0.5	0.5	0.6	0.5
α-Humulene	0.1	0.2	0.3	0.1	0.1	0.1
Germacrene D	0.1	0.1	0.1	0.1	0.1	0.1
δ-Cadinene	1.2	1.1	1.2	1.1	1.2	0.9
TOTAL	**100**	**100**	**100**	**100**	**100**	**100**
Diterpenes						
Palmitic acid	3.6	3.9	2.6	3.0	2.5	2.3
Abienol	9.2	9.1	8.8	9.0	9.3	6.8
Pimaric acid	8.7	7.6	8.3	9.5	9.0	6.6
Sandaracopimarate	10.8	9.2	10.0	11.8	10.7	8.0
Levopimaric acid	**31.6**	**31.6**	**35.2**	**33.2**	**32.0**	**49.1**
Dehydroabietic acid	12.6	15.2	12.8	11.8	15.2	8.8
Abietic acid	9.1	9.1	9.3	8.7	9.1	8.0
Neoabietic acid	9.1	8.4	9.0	9.0	8.6	7.6
TOTAL	**100**	**100**	**100**	**100**	**100**	**100**
Wood Phenolics						
Catechin	2.9	3.8	3.3	1.7	1.4	1.8
Epicatechin	0.0	0.0	0.0	3.0	0.0	0.0
Astringin	20.2	27.3	16.7	29.7	28.6	23.6
Piceid	5.1	4.9	2.4	3.9	9.4	2.7
Isorhapontin	71.8	62.9	76.1	56.7	60.7	71.9
Piceatannol	0.0	1.1	1.5	5.0	0.0	0.0
TOTAL	**100**	**100**	**100**	**100**	**100**	**100**
Bark Phenolics						

Unknown	0.9	1.1	1.3	1.1	1.2	1.3
Catechin	2.4	1.8	2.0	2.6	2.8	3.1
Epicatechin	2.0	1.9	2.0	2.0	1.6	2.2
Astringin	38.8	38.3	40.6	41.1	41.3	39.0
Unknown	0.8	1.0	0.4	0.5	0.7	1.3
Piceid	7.7	6.8	7.8	6.4	7.1	6.8
Isorhapontin	46.5	48.4	44.5	45.5	44.5	44.8
Unknown	0.3	0.1	0.1	0.1	0.1	0.1
Piceatannol	0.4	0.2	0.2	0.1	0.1	0.5
Unknown	0.3	0.4	0.9	0.7	0.5	0.9
TOTAL	**100**	**100**	**100**	**100**	**100**	**100**

3.1.6. Methyl jasmonate increases *P. abies* resistance to *C. polonica*

Forty-year-old *P. abies* that had been pretreated with methyl jasmonate were much better protected against subsequent mass inoculation with *C. polonica* than were untreated control trees (Figure 3.12). Even the lowest concentrations of methyl jasmonate (5 and 25 mM) caused a significant decline in fungal colonization of sapwood (Figure 3.12) with blue-staining declining from 70 % in untreated controls to 40 % at 5 mM methyl jasmonate. Fungal staining was extremely attenuated at the higher concentrations: 15 % at 50 mM and less than 1 % at 100 mM. Methyl jasmonate had less dramatic effects on cambium necrosis, but the two highest concentrations also gave significant reductions in symptoms (83% reduction relative to control for 100 mM; Figure 3.12). The six clones differed significantly in their response to methyl jasmonate treatment and fungal inoculation (blue-stained sapwood: $F = 15.11$, $P < 0.0001$; necrotic cambium: $F = 6.85$, $P = 0.0004$). Clone 63 showed no response to MJ treatment (100% blue-stained sapwood and necrotic cambium at all concentrations, including the control), whereas clone 85 responded to even the lowest concentrations. The other clones had intermediate responses. Data for clone 63 were included in the statistical analyses but are not presented in Figure 3.12.

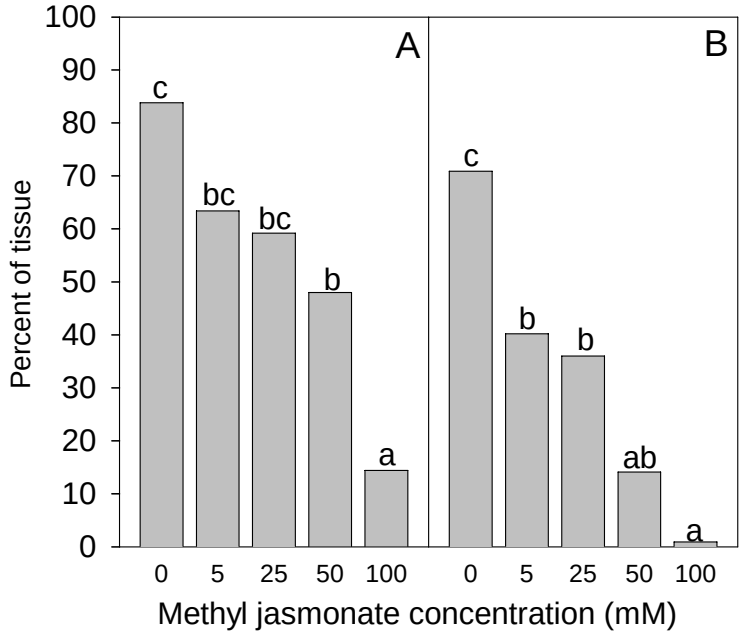

Figure 3.12. Symptoms of fungal infection in *Picea abies* pretreated with various concentrations of methyl jasmonate: (A) percentage dead cambium by circumference, (B) percentage blue-stained sapwood. Mass inoculation of *Ceratocystis polonica* was done four weeks after methyl jasmonate application and symptoms were measured 15 weeks after inoculation. Bars with the same letter were not significantly different (LSD test a P=0.05 following ANOVA).

3.2 Methyl jasmonate, terpenes and resistance to *Ips typographus*

3.2.1. Methyl jasmonate increased the number of traumatic resin ducts and the accumulation of terpene resin constituents

The *P. abies* trees used for this study were much older (60 vs. 30 years old) than in the *C. polonica* study described in section 3.1, because trees younger than 50 years old are not naturally attacked by *I. typographus* due to the paucity of phloem tissue necessary to support larval growth. Anatomical analyses of these 60-year-old trees showed that in both years there were significantly more traumatic resin ducts (TDs) in the xylem of MJ-treated sections of trees than in the xylem of untreated control sections (2003: 27.7% vs. 1.8% of sapwood circumference, P = 0.001; 2004: 14.2 vs. 5.9%, P = 0.04; one-sample t-test). There was no significant difference in TD abundance in MJ-treated sections between years (P = 0.11, t-test). The concentrations of monoterpenes, diterpenes, and total terpenes were significantly higher in

MJ-treated bark and sapwood than in control tissues (Figure 3.13). This was true both for the year of MJ application (2003) and the following year, but the response was much weaker in 2004, particularly in the bark (Table 3.2).

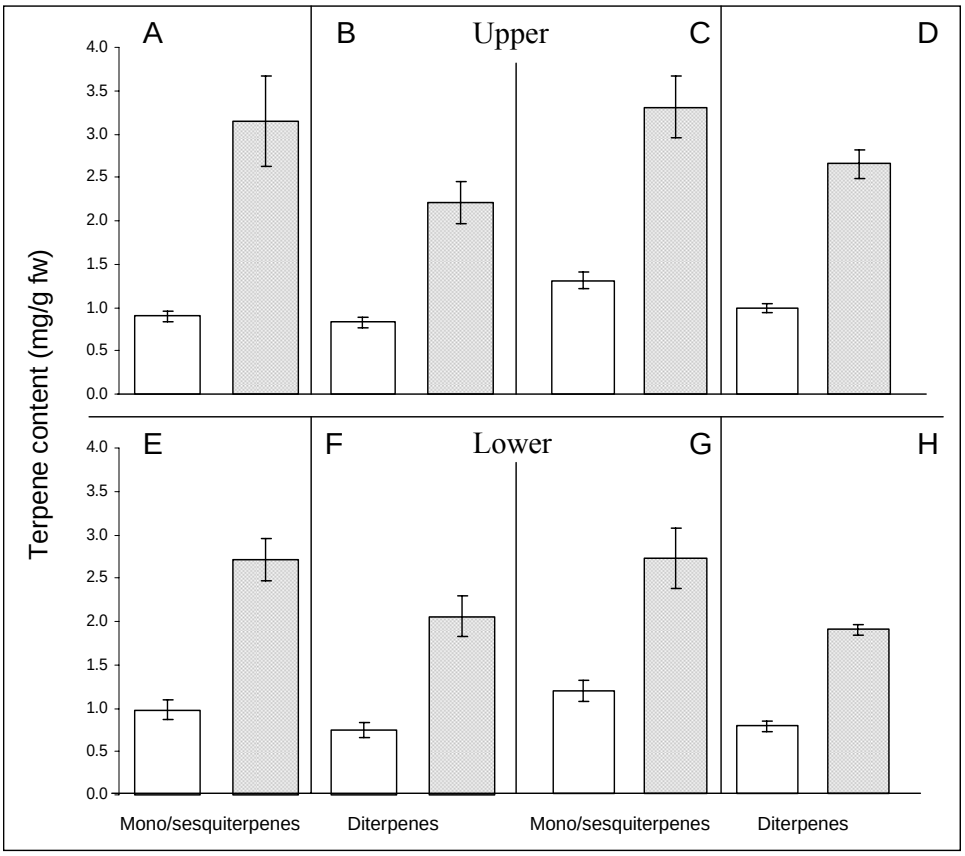

Figure 3.13. Effect of methyl jasmonate (MJ) on terpenes of *P. abies* during the year of treatment (2003) at two different heights above the ground. Untreated controls = white bars; MJ treatment = gray bars. (A), (B), (E) and (F) = wood samples; (C), (D), (G) and (H) = bark samples.

Total terpene concentration in bark was 2.5 fold higher after MJ treatment in 2003 than in untreated portions of trees, but only 1.3 fold higher in 2004. In wood, the corresponding fold-differences were 3.0 and 2.1. There were no qualitative differences in terpene composition in MJ_C vs. MJ-treated bark or wood ($R^2 > 0.99$ for linear regression of percent composition of individual terpenes in MJ_C and MJ-treated tissues). A total of 27 different terpenes were detected with the monoterpenes, α- and β-pinene and limonene, the sesquiterpene germacrene D, and the diterpenes, dehydroabietic acid, isopimaric acid, and neoabietic acid, making up nearly three-quarters of the total terpenes. The total amount of terpenes was roughly the same in the bark and wood (Table 3.2).

Table 3.2. Effect of methyl jasmonate (MJ) treatment on terpene and phenolic content (mg g^{-1} fresh weight of tissue) of Norway spruce trees during the year of treatment (2003) and the following year (2004). Treatments were compared using one-sample t-test (n = 11 in 2003, 12 in 2004).

	2003				2004		
	Control	MJ	*p*		Control	MJ	*p*
Bark:							
Total monoterpenes	0.94	2.33	< 0.001		1.22	1.48	0.037
Total sesquiterpenes	0.19	0.49	0.085		0.21	0.19	1.000
Total diterpenes	0.82	2.02	< 0.001		1.11	1.67	0.012
Total terpenes	1.96	4.84	< 0.001		2.54	3.35	0.016
Total soluble phenolics	33.98	29.40	0.780		23.48	26.29	0.540
Wood:							
Total monoterpenes	0.83	2.42	< 0.001		1.13	2.22	< 0.001
Total sesquiterpenes	0.11	0.48	0.014		0.17	0.29	0.042
Total diterpenes	0.76	2.13	< 0.001		0.92	2.06	< 0.001
Total terpenes	1.71	5.04	< 0.001		2.21	4.56	< 0.001
Total soluble phenolics	0.61	0.50	0.800		0.28	0.32	0.240

HPLC analysis of the crude methanol extracts revealed that there were many more soluble phenolics in the bark than in the wood, both in terms of number of individual compounds detected and total quantities. The stilbene glycosides astringin and isorhapontin dominated, making up about 67% of the total soluble phenolics in the bark and 50% in the wood. However, there were no quantitative differences in phenolic content between MJ-treated bark or sapwood and untreated tissue on the same tree (MJ_c), neither for individual compounds ($P > 0.22$, one-sample t-test) (Figure 3.14) nor for total soluble phenolics (Table 3.2).

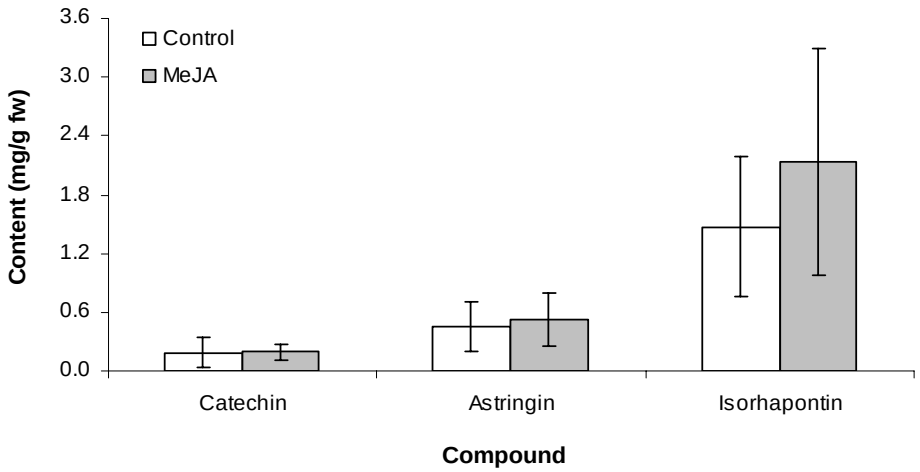

Figure 3.14. Effect of methyl jasmonate (MJ) treatment on the levels of the major soluble phenolic compounds.

Nor were there any qualitative differences in soluble phenolic composition between MJ-treated and untreated tissues (R^2 = 0.85 for wood and 0.99 for bark, linear regression of percent composition of individual phenolics in MJ_C and MJ-treated tissues).

3.2.2. Methyl jasmonate (MJ) reduced *Ips typographus* colonization of *P. abies* bark

After MJ treatment of portions of the bark surface of twelve 60-year-old *P. abies*, the attack of *I. typographus* was induced three weeks later by pheromone dispensers attached to the trees. Although one tree was mass-attacked and killed in 2003, attacks on the other 11 trees were all aborted. No tunnels extended more than 50 mm from the entrance hole in the eleven surviving trees and no oviposition took place; this allowed comparison of the number and length of attacks between MJ-treated and untreated bark without considering their success. The application of the pheromone was very effective and beetle attacks did not extend far from the pheromone dispenser. Nearly all were confined to the sampling area.

MJ-treated bark suffered much less bark beetle colonization than did untreated bark on the same tree (referred to as "MJ_c"), both at the lower and upper sampling positions (Figure 3.15A,E; P= 0.0001-0.05). Treated bark had an average of 31 % fewer entrance holes and 69 % fewer galleries (Figure 3.15A,E), and gallery length was 82 % shorter than in untreated bark

(Figure 3.15B,F). All of these differences were statistically significant (Figure 3.15A,B,E,F). The difference between MJ_C and MJ-treated bark was significantly greater with regard to total gallery length (MJ_C:MJ ratio of 6.53) than to number of incipient galleries and entrance holes (MJ_C:MJ ratios of 2.40 and 1.66, respectively (F = 4.48, P = 0.015). This suggests that the negative impact of MJ increased as the beetles proceeded along the colonization sequence from first entry into the bark to sustained tunneling activity. Beetle colonization also varied significantly with sampling position, with more colonization on the lower position, which was closer to the pheromone source (F = 4.24-13.75, P = 0.0006-0.04 for the different colonization variables).

These lower samples were probably more influenced by proximity to the pheromone dispenser itself, and thus the upper samples probably offer a more unbiased view of beetle attack behavior. However, the effect of MJ treatment (measured as the difference between MJ_C and MJ-treated bark within each tree) did not differ between sampling positions (F = 0.01-2.06, P = 0.17-0.94 for the different colonization variables). One year after MJ treatment, *I. typographus* attacks were again induced by attaching pheromone dispensers on the eleven surviving trees. Although the number of beetle attacks in 2004 was much lower than in 2003 (115 vs. 434 attacks/m^2, respectively; F = 82.51, P < 0.0001), the 2004 attacks were much more successful in terms of total gallery construction (4.63 vs. 1.47 m tunnel/m^2; F = 2.78, P = 0.10) (Figure 3.15C,G).

MJ treatment had no significant effect on beetle colonization in 2004 when the whole data set was analyzed (P = 0.16-0.40 for the different variables; one-sample t-test). However, if one tree with extensive beetle colonization on the MJ treated half of the tree is excluded from the analysis, MJ-treated bark once again had significantly fewer galleries (56.85 vs. 82.35 per m^2, P = 0.021) and less total gallery length (3.51 vs. 5.82 m per m^2, P = 0.048) (Figure 3.15D,H) than control bark did. At any rate, these differences were much smaller than those observed between MJ-treated and MJ_C bark after the 2003 attacks (galleries: 41.54 vs. 117.33 per m^2; total gallery length: 0.58 vs. 2.37 m per m^2).

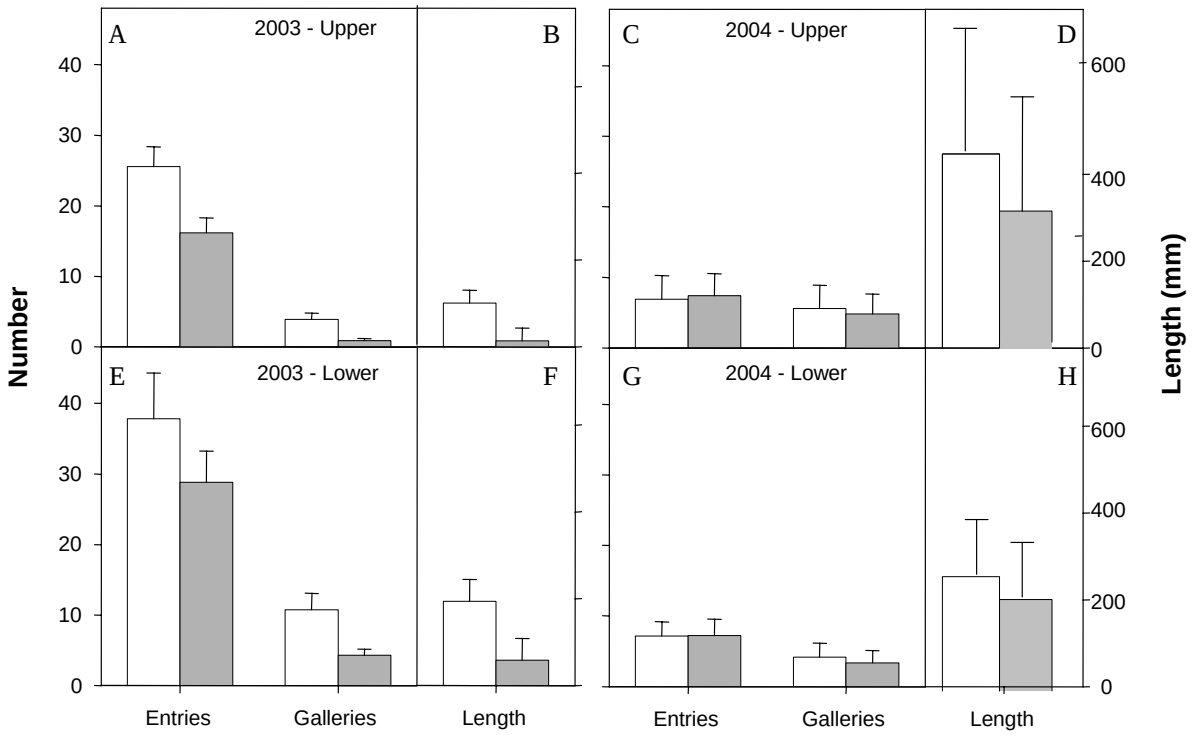

Figure 3.15. Effect of methyl jasmonate (MJ) on *I. typographus* colonization of *P. abies* during the year of treatment (2003) and the following year (2004) at two different heights above the ground. Untreated controls = white bars; MJ treatment = gray bars. Entries = number of beetles entering the bark, galleries = number of initiated egg galleries, length = total gallery length. Bars = 1 SE, n = 11 in 2003 and 12 in 2004.

In the Petri dish assays, the number of beetle entrance holes in fresh bark discs did not vary among treatments, suggesting that MJ application did not affect host entrance by *I. typographus* (Table 3.3). However, in these assays MJ had significant effects on beetle behavior within the bark; control bark had a significantly higher number of parental galleries than MJ-treated bark, gallery length was longer, and more eggs were deposited (Table 3.3). There were no significant differences between discs from untreated parts of MJ-treated trees and those from completely untreated trees. The male:female ratio in each assay unit was similar among treatments ($F_{(2,24)}$ = 1.09, *P=0.367* sex by treatment interaction), and ranged from 0.43 to 0.5.

Table 3.3. Bark beetle colonization of Norway spruce bark treated with methyl jasmonate (MJ) and untreated bark (Control = bark from untreated tree; MJ_C = untreated part of MJ treated tree). Means followed by the same letter in a column are not significantly different at $P< 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data (square root√y). Non-transformed means ± SE are reported.

	No. entrance tunnels	No. galleries	Gallery length (cm)	No. egg niches
Control	5.47 ±0.26	3.60 ±0.35 a	19.83 ±2.86 a	36.67 ±8.65 a
MJ_C	5.13 ±0.32	4.07 ±0.48 a	22.30 ±2.25 a	45.53 ±6.30 a
MJ	5.53 ±0.24	2.47 ±0.31 b	9.33 ±1.29 b	10.80 ±3.10 b
$F_{(2,12)}$	0.71	5.55	17.48	14.31
P	**0.5125**	**0.0196**	**<0.0001**	**0.0007**

3.2.3. Methyl jasmonate reduced *Ips typographus* aggregation

Attraction of *I. typographus* to conspecifics that had colonized MJ-treated or untreated bark was tested by attaching Petri dishes containing beetles in bark discs to flight interception traps. A total of 273 bark beetle specimens were captured in these traps across treatments, including 170 *I. typographus* (89 female, 81 male), 25 *Hylastes cunicularius*, 43 *Dryocoetes autographus,* and 35 *Pityogenes chalcographus*. Use of MJ-treated bark discs significantly reduced the number of beetles attracted, as male *I. typographus* excavating in MJ-treated bark attracted fewer conspecifics than did males excavating in MJ_C bark (untreated bark from trees that had been treated elsewhere with MJ) or control bark (bark from untreated trees) (Table 3.4). There was also a significant effect of time ($F_{(2,24)}$ = 30.97, P < 0.0001) and time by treatment interaction ($F_{(4,24)}$ = 3.23, P =0.03) on the number of beetles collected. This was probably due to variable weather conditions with frequent rainfall during parts of the trapping period. There was a significant correlation between the number of beetles attracted and mean gallery length in the bark discs (P = 0.005, R^2 = 0.67). The proportion of male vs. female *I. typographus* beetles attracted to the traps (averaging 47% male) was not affected by MJ treatment ($F_{(1,24)}$=0.79, P =0.383 for sex and $F_{(2,24)}$=0.98, P =0.39 for sex by treatment interaction). *Pityogenes chalcographus* showed a similar response pattern as *I. typographus* (Table 3.4). For *H. cunicularius* and *D. autographus* trap catches did not appear to differ

among treatments. No time or time by treatment effects were observed in any of the latter three species.

Table 3.4. Response of flying bark beetles to male and female *I. typographus* tunneling in Norway spruce bark treated with methyl jasmonate (MJ) and untreated bark (Control = bark from untreated tree; MJ_C = untreated part of MJ-treated tree). Means (number of insects caught per trap and 5-day collection period) followed by the same letter in a column are not significantly different at $P< 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data (square root√y). Non-transformed means ± SE are reported.

	Number of insects of each species trapped			
	I. typographus	*P. chalcographus*	*D. autographus*	*H. cunicularius*
Control	5.00 ±0.82 a	1.33 ±0.44 a	1.20 ±0.29	**0.73 ±0.18**
MJ_C	5.00 ±1.08 a	0.87 ±0.38 a	0.93 ±0.43	**0.60 ±0.27**
MJ	1.33 ±0.33 b	0.13 ±0.09 b	0.73 ±0.27	**0.33 ±0.19**
$F_{(2,12)}$[1]	13.72	4.09	0.33	**0.86**
P	**0.0008**	**0.044**	**0.723**	**0.448**

[1] *Degrees of freedom: Numerator degrees of freedom and denominator degrees of freedom*.

3.2.4. Methyl jasmonate reduced Ips typographus reproduction in detached logs

In another experiment, bolts cut from trees that had been treated with MJ or were untreated were placed in a *P. abies* forest and left to be colonized by *Ips typographus* attracted by pheromone dispensers. Beetle reproduction was negatively affected by MJ treatment. Significant reductions occurred in total numbers of beetles produced per dm^2 bark surface, mean dry weight per beetle, and total beetle dry weight per dm^2 bark when beetles colonized bolts cut from MJ-treated sections (Table 3.5, one pair of logs with aberrant results was omitted from the analysis).

Table 3.5. Mean number of *I typographus* emerged per dm^2 bark surface, mean dry weight (dw) per beetle, and total beetle dw per dm^2 bark surface on logs treated with methyl jasmonate (MJ) and untreated logs (Control = untreated log; MJ_C = untreated half of MJ-treated log). Means followed by the same letter in a row are not significantly different at $P< 0.05$, based on Proc Mixed and Tukey's Protected LSD test on transformed data (square root√y). Non-transformed means ± SE are reported.

	No. beetles/dm^2	Mean dw/beetle (mg)	Total beetle dw/dm^2
Control	43.61 ±3.37 a	6.17 ±0.13 a	267.66±19.86 a
MJ_C	42.36 ±3.02 ab	5.87 ±0.18 a	249.08±19.85 ab
MJ	35.11 ±3.31 b	5.17 ±0.12 b	182.98 ±19.62 b
$F_{(2,20)}$	5.54	13.1	6.53
P	**0.012**	**0.0002**	**0.006**

3.3 Methyl jasmonate, tepenes and resistance to *Pissodes strobi*, the white pine weevil

The experiments described in this section employed 15-year-old field grown Norway spruce trees, since they can support the white pine weevil life cycle and their size permitted injection of jasmonic acid (JA). The work not only investigated the effect of methyl jasmonate treatment on resistance to *P. strobi*, but also compared the changes in terpene content caused by *P. strobi* feeding with that caused by mechanical damage.

3.3.1. Effects of jasmonate, weevil feeding and mechanical wounding on terpenoid accumulation

To test if feeding by white pine weevils could induce any changes in terpenoid resin composition or accumulation, compared to mechanical wounding or jasmonic acid, we analyzed the needles and bark of developing apical shoots after all three treatments.

All trees contained the same basic complement of monoterpenes and sesquiterpenes, but differed in the relative amounts of some of these substances. We found slight differences in constitutive terpenes between bark and needles (Figure 3.16) which is in agreement with

previous findings that terpene concentration and composition depend on the tissue, time of sampling, and geographical location. Needles have more monoterpenes and sesquiterpenes than does bark tissue. The major difference in the composition is the presence in relatively high aboundance of bornyl acetate in needles which is less than 1% in bark tissue. However, the monoterpene fraction in all cases was dominated by α- and β-pinene, although their pre-treatment concentrations (constitutive levels) varied between clones. These two terpenoids alone accounted for ca. 65-70% of the total monoterpene content, while about 20% of the total monoterpene content consisted of a mixture of δ-3-carene, myrcene, limonene, and β-phellandrene. Sesquiterpenes constituted only a minor part of the total terpenes and are represented only by four compounds, namely, α-humulene, longifolene, germacrene, and δ-cadinene. The diterpene fraction was dominated by levopimaric acid and abietic acid, accounting for ca. 55-60% of the total diterpenes. Although some of the sesquiterpenes acids can exist as different enantiomers, we analyzed only the enantiomeric composition of the monoterpene fraction, as this constituted over 95% of the total volatile fraction. Our study revealed the presence of a high percentage of (-)-enantiomers of β-pinene and limonene. In the decreasing order, the main constituents were (-)-β-pinene, (+)-α-pinene, (-)-α-pinene, (-)-limonene, and (+)-limonene. The level of (-)-α-pinene was found to be very variable. However no changes in the ratio between (-) and (+) enantiomers were observed.

However, several differences in the responses of the bark and needles between the insect and jasmonic acid treatments were found (Figure 3.16). In the bark tissue, feeding by the white pine weevil induced a near 2 fold increase in monoterpenoid and sesquiterpenoid content (Figure 3.16B), but no significant changes in the proportion of the different diterpenes in the oleoresin (Figure 3.16D). Mechanical wounding also caused an increase in monoterpenoid but not sesquiterpenoid accumulation. In the needles, both weevils and jasmonic acid, but not wounding, caused an increase in terpenoid accumulation of almost 2-fold for volatile fraction and 3-fold for the non-volatile fraction 32 days after the treatments began (Figure 3.16A and 3.16C).

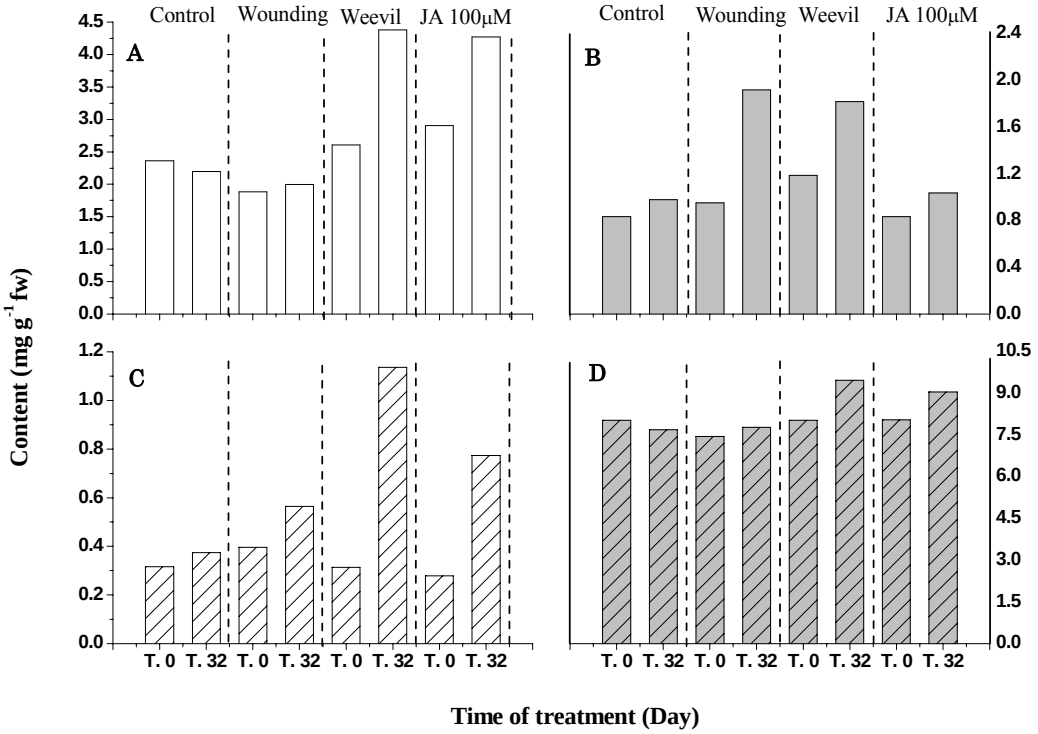

Figure 3.16. Effect of different treatments on: (A) needle mono/sesquiterpene content, (B) bark mono/sesquiterpene content (C) needle diterpene content, and (D) bark diterpene content. Terpene content was measured just prior to treatments (T.0) and 32 days later (T. 32).

The strongest terpenoid accumulation in both bark and needles was recorded 32 days after the treatments began. Although trees responded on a different scale to the different stimuli, it seems that the time-course of responses was similar, suggesting that the same mechanism is involved after JA injection (or its methyl ester) or mechanical wounding, or after white pine weevil feeding in Sitka spruce. In general, the effect of treatments on the production of terpenes in conifers seems to be quantitative rather than qualitative, in that the response of the tree is characterized by changes in the relative concentrations of most of the bark terpenes rather than induction of the novel terpenes.

3.3.2. Effect of treatments on formation of traumatic resin ducts

Application of jasmonic acid, mechanical wounding, and weevil feeding induced *de novo* differentiation of traumatic resin duct cells. Resin ducts are considered to be a characteristic part of the *Pinaceae* constitutive defenses but are in relatively low numbers during the normal growth. Our results demonstrate that traumatic resin ducts form in the xylem following injection of jasmonic acid (Figure 3.17A), mechanical wounding (Figure 3.17B), or white pine weevil attack (Figure 3.17C). It has been suggested that traumatic resin duct formation in xylem is associated with the defense responses of spruce trees toward invading insects, namely, that the resin, originating from traumatic or constitutive ducts, could flood the oviposition cavities and thus kill the weevil eggs and larvae. However, as previously noted, the white pine weevil's performance was apparently unaffected by these induced changes. Nonetheless, it has always been difficult to understand how the xylem traumatic ducts could influence weevil performances as this insect mainly attacks the bark of its hosts. Clearly, a connection exists between these axial traumatic resin canals and the constitutive radial ones located in rays. This network of resin canals would contribute to the flow of resin that occurs after bark injury, with some resin likely coming directly from the xylem where the producing epithelial cells remain active and protected from the weevil damage that is mostly limited to the bark during weevil attack.

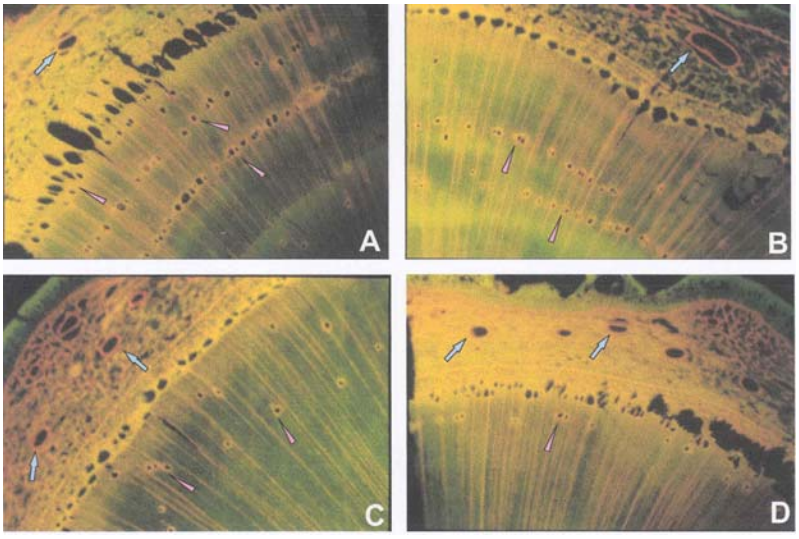

Figure 3.17. Cross-section of stems from 15-year-old Norway spruce terminal leaders 60 days after (A) jasmonic acid injection, (B) mechanical wounding, (C) white pine weevil attack, and (D) water

injection treatments. Figures include periderm, cortex, phloem, cambium and xylem. Symbols: ⇨ = constitutive resin duct, Δ = traumatic resin duct. Magnification: 36X

The increase in the total production of terpenes is correlated with an increase in the number of traumatic ducts. Although our sampling procedure makes it difficult to determine whether the additional terpenes induced after treatment were translocated to the growing leaders or were *de novo* synthesized in the treated stem, the time course for the differentiation of traumatic resin ducts and the accumulation of terpenes in the newly formed resin ducts strongly suggest that they were synthesized *de novo*.

3.3.3. Effects of treatments on white pine weevil performances

After showing that resistance to bark beetles and a blue-stain fungus can be stimulated by spraying spruce trees with MJ, we were interested to see if JA injection or wounding could alter white pine weevil behavior and biological performance. However, in the present study, the applied treatments had no impact on the white pine weevil biological performance (Table 3.6). Neither JA injection nor wounding treatments had an effect on white pine weevil feeding activity ($F_{4,49} = 0.72, P = 0.5854$), oviposition ($F_{4,49} = 0.61, P = 0.6599$) or number of pupal chambers ($F_{4,49} = 1.58, P = 0.1948$).

Table 3.6. Variance analysis for the number of feeding punctures, oviposition holes, and pupal chambers observed after 60 days of development on Norway spruce terminal leaders following either jasmonic acid injection (10 μM and 100 μM), water injection, mechanical wounding, or control (with only three mated white pine weevil females).

Parameter evaluated	df treatment	df error	F value	Pr > F
Feeding punctures	4	49	0.72	0.5854
Oviposition holes	4	49	0.61	0.6599
Pupal chambers	4	49	1.58	0.1948

3.4. The use of cell cultures to study defensive terpene formation in spruce

To understand the complex regulation of terpenoids in spruce, the signaling pathways involved and of the flux through the various pathways during the normal growth cycle and following elicitation, we took advantage of the fact that suspension cultured cells exhibit many of the responses that are characteristic of intact tissues in certain aspects of induced terpenoid metabolism and regulation.

3.4.1. Growth of cultured cells

The first task was to characterize the growth of cells in culture; several lines of undifferentiated cells were tested that had originally been started from embryos. A different rate of cell growth for the different lines was observed under normal conditions (nonelicited) (Figure 3.18). Growth rate of the lines was determined on the basis of mg cell per mL media Cell line *Pa.* M1 had the highest growth rate, while line *Pa.* M 2 was the slowest.

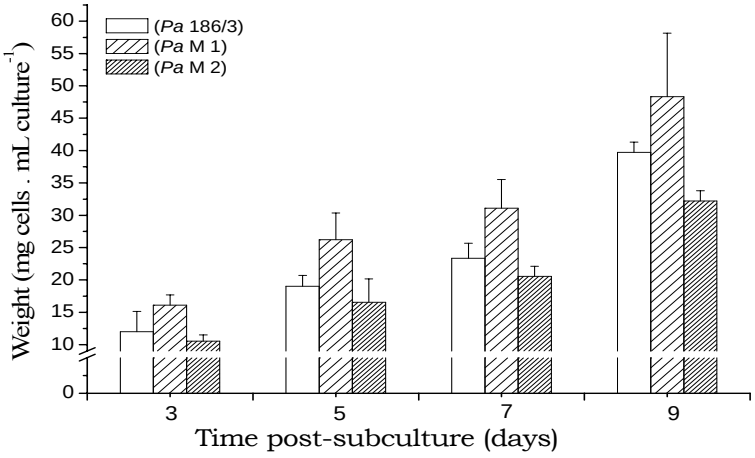

Figure 3.18. Growth dynamics of *Picea abies* cell line suspension cultures. Data are presented as mean ± standard error (N =3).

3.4.2. Monoterpene production in cultured cells

By optimizing the method of monoterpene extraction (see methods), the concentration of the elicitor, and the timing of elicitation, we could show for the first time

the endogenous production of terpenoids in *Picea* cell suspension cultures, in contrast to previous reports (Lindmark-Henriksson et al., 2004). We found that suspension cultures constitutively accumulated small amounts of monoterpenes with a product profile similar to that of adult trees (Figure 3.19). However, the greatest accumulation of monoterpenes occurred when elicitors were added to cultures at a final concentration of 50 µM (methyl jasmonate) or 100 µg/mL (chitosan) on day 3 of the 10 day-culture cycle. Elicitation occurred only in one cell line, *Pa.* 186.3. Our study revealed that *Pa.* 186.3 cells accumulated α-pinene, β-pinene and limonene in trace amounts. The other monoterpenes of mature trees were not present in the culture. While this may simply reflect varietal differences, it may also underscore subtle differences in the expression of members of the spruce monoterpene synthase gene family in culture. Sesquiterpenes and diterpenes, although present in spruce saplings and trees, were also not detected in these cell suspension cultures. Monoterpene production was induced similarly by methyl jasmonate and fungal cell walls and fragments thereof (chitosan). Elicitation with methyl jasmonate caused a 3-4 fold increase in monoterpene production compared with control (unelicited) (120 µg/g culture *vs* 35 µg/g). Chitosan elicitation was weaker than MJ, causing an increase 2-2.5 fold compared with unelicited controls (80 µg/g culture vs 35 µg/g). Culture age, relative to the most recent transfer, slightly affected the constitutive level of monoterpenes.

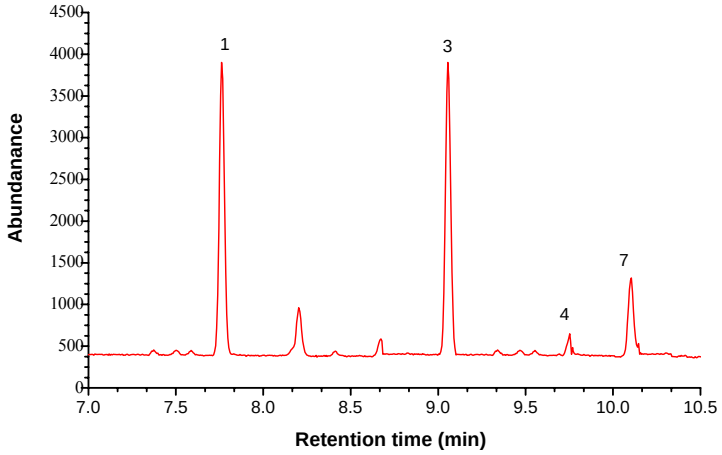

Figure 3.19. Endogenous monoterpene production in spruce suspensions cultures extracted after incubation with XAD-4 resin. Numbers of the compounds refer to the Figure 3.2.

All of the major monoterpenes present in the elicited culture were also present in the nonelicited culture, but the relative proportion of the monoterpenes was different, indicating that elicitation does not result in the production of new types of monoterpenes, but simply affects the relative abundance of existing terpenes.

Induction of secondary pathways in plant cell cultures can sometimes be triggered by exposing the cultures to abiotic stress, i.e. to limitation of specific nutrients in media, variation on the nutrient composition of the growth medium, changes in the medium pH, etc. To determine if pH could alter terpene accumulation, we measured the pH of the liquid in the cell-free medium under different elicitor treatments. Addition of 100 µg/mL chitosan (pH 6.5) caused an increase in the pH of the medium to a level higher than control or MJ elicited cell cultures (Figure 3.20).

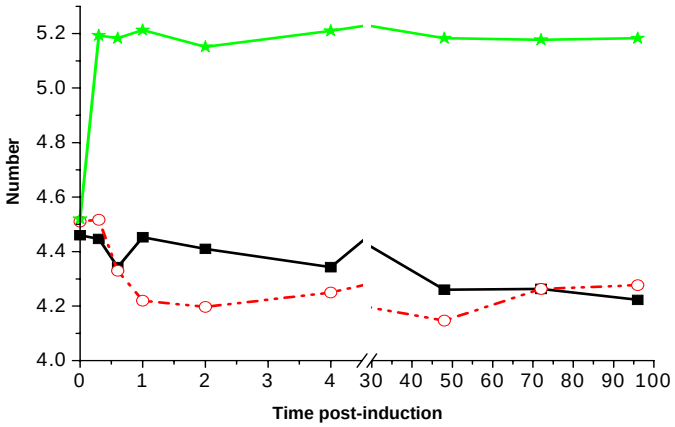

Figure 3.20. Effect of treatment on the medium pH. The cells were incubated in the production medium and treated with chitosan (100 µg/mL) or methyl jasmonate (MJ, 50 µM). Cells receiving water were used as a control. Data represent means±standard deviations from three independent experiments. Untreated controls = solid black line; MJ treatment = red dashed line; Chitosan treatment = green line.

To test if these pH differences affect the cell growth, the growth rate of the cultured cells was determined on a weight per volume basis. Culture density increased as cell cultures aged, but this was independent of the treatment. Whereas there appeared to be a 5-10% decrease in cell growth between control flasks and flasks elicited with 50 µM methyl jasmonate or chitosan (Figure 3.21), these differences were not significant due to the low number of replicates (P= 0.63).

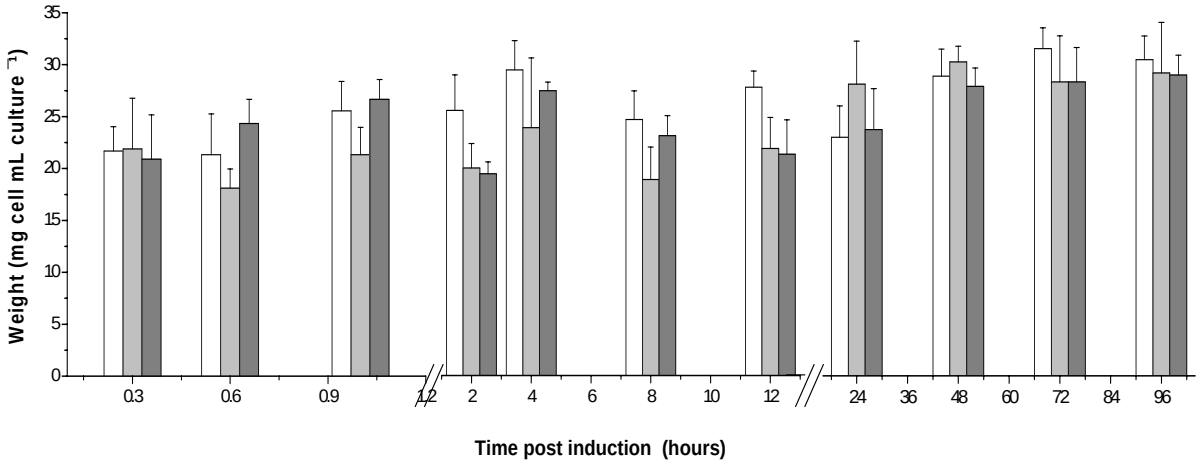

Figure 3.21. Effect of treatment on growth dynamics of *Picea abies* suspension cultures. Untreated controls = white bars; MJ treatment = light gray bars; Chitosan treatment = dark gray bars. Data are presented as mean ± standard error (N =3).

3.4.3. Monoterpene biosynthetic activity in cultured cells

The enhanced accumulation of monoterpenes in culture was paralleled by a several fold increase in monoterpene synthase activity as measured by *in vitro* enzyme assays (Figure 3.22). Compared to non-induced controls, MJ and chitosan induced cultures showed a lasting, significant increase in monoterpene synthase activity within few hours of treatment (Figure 3.22). The increase was noticed as early as 20 minutes after induction compared with nonelicited controls. When chitosan was used as elicitor, monoterpene synthase reached the maximum activity at 12 hours post-inoculation, decreasing slightly after that. The pattern of monoterpene synthase activity elicited by MJ was similar to that of chitosan. Maximum activity was not reached until 48 to 72 hours post-inoculation (Figure 3.22). Culture age, relative to the most recent transfer, had a slight negative effect on the ability of the cells to produce terpenes and elevate monoterpene synthase activity. The activity of monoterpene synthases in nonelicited cells was almost constant and slightly increased 7-8 days post-subculture (Figure 3.22).

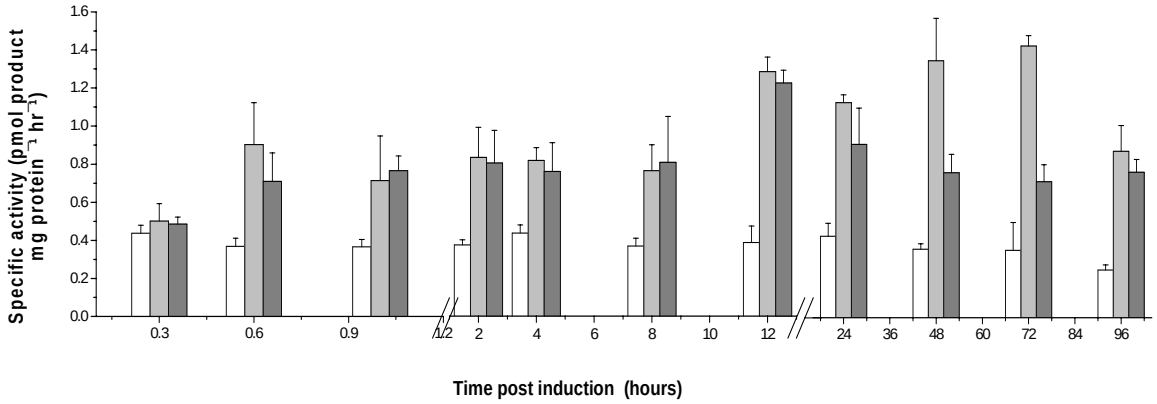

Figure 3.22. Kinetics of monoterpene synthase activity from *Picea abies* suspension cultures (line *Pa.* 186.3). Untreated controls = white bars; MJ treatment = light gray bars; Chitosan treatment = dark gray bars. Data are presented as mean ± standard error (N =3). The cells were incubated in the production medium and treated with chitosan (100 µg/mL) or methyl jasmonate (MJ, 50 µM). Cells receiving water were used as a control.

DISCUSSIONS

Defenses induced by herbivores and pathogens are widespread in the plant kingdom, and are thought to be favored if the incidence of attack is uncertain and the costs of producing and storing defenses are high (Karban and Baldwin, 1997). However, our knowledge of induced defenses in woody plants is vastly inferior to that in herbaceous species. I attempted to manipulate the defenses of a model woody plant, Norway spruce (*Picea abies*), in order to test the protective roles of various substances. Induced defenses could be important in *Picea abies* and other forest trees since the pattern of insect attack can be very variable from year to year as evidenced by the occurrence of *Ips typographus* outbreaks (Wallner, 1987; Christiansen and Bakke, 1988; Økland and Berryman, 2004). Among the major types of induced defenses produced by *Picea abies* are terpenes and phenolics among others, in response to herbivore damage or pathogen infection. To alter the defenses of *P. abies* in a non-invasive way, we chose to apply jasmonates, which are major regulators of plant defense metabolism in angiosperms that have recently been shown to have effects on cell cultures and young saplings of gymnosperms (Kozlowski et al., 1999; Martin et al., 2002, Ketchum et al., 2003; Heijari et al., 2005; Huber et al., 2005). Application of methyl jasmonate or jasmonic acid to mature *Picea abies* trees induced many of the complex chemical and anatomical defenses normally triggered by enemy attack, allowing us to test their roles in defense against two insects species and an insect-associated fungus. In the discussion, we first consider how jasmonate treatment changed the amount of defenses and defense structures, and then go on to elaborate on how jasmonate treatment altered resistance to insect and fungal attack.

4.1. Methyl jasmonate induces resin duct formation

Resin ducts are major defensive structures found in nearly all organs of the *Pinaceae.* In the stem, these include radial resin ducts derived from radial rays, axial resin ducts or canals, resin blisters and resin cells. Axial resin ducts are common in the secondary phloem of the *Pinaceae* (Franceschi et al., 2005), and are considered to be a characteristic part of their constitutive defenses, but are found in relatively low numbers during normal growth although their density (number of canals related to wood increment) is known to be influenced by climatic conditions (Wimmer and Grabner, 19997)

Within a month following methyl jasmonate (MJ) treatment, the formation of a new row of resin ducts in the sapwood (the newly developing xylem) was observed. Formation of these traumatic ducts was also observed following injection of jasmonic acid, mechanical wounding and *P. strobi* attack. This process disrupts the normal vascular development, involving complex changes in cell division patterns and differentiation.

The anatomical observations on trees treated with MJ, JA, mechanically wounded, or damaged after weevil feeding indicate a time-dependent induction process in traumatic duct formation, in which a signal originating at the infection site propagates up the stem and induces differentiation of mother xylem cells into traumatic duct. This finding is consistent with recent evidence for a similar phenomenon in Austrian pine (*Pinus nigra* Arn.) (Luchi et al., 2005), in which a putative signal triggers the propagation of a developmental wave in the axial direction. This evidence, suggests that the formation of traumatic ducts in *P. abies* is mediated by endogenous signals. In the signalling cascade leading to traumatic ducts formation, MJ probably acts through ethylene as an intermediary (Hudgins and Franceschi, 2004).

Application of MJ was associated with a high increase in resin flow. On average, resin flow after MJ treatment was triple that in untreated trees demonstrating that the traumatic ducts produced in response to treatements were actually functional. Numerous cytological, physiological and molecular biological studies of conifer species indicate that the terpenes secreted into developing resin ducts or resin blisters are newly formed in the surrounding epithelial cells (e.g. Lewinsohn et al., 1991a; Miller et al., 2005). Once formed, terpenes can be translocated from their site of origin via the interconnected network of vertical and radial resin ducts found in *Picea abies* and other species of conifers (Franceschi et al., 2000). This is probably the explanation for the extensive resin flow observed on the outer bark of methyl jasmonate-treated trees which were significantly higher than on the bark of control trees. Resin from newly-formed traumatic ducts moves via radial ducts to the trunk surface. Once on the bark, it may serve as an initial barrier against pathogen and herbivore invasion. Increased resin flow can help to kill or flush out invaders as well as seal the wound, and resin-soaked regions of bark and sapwood may also be more resistant to introduced microbial activity. The increase in resin flow in spruce after methyl jasmonate application was slower than previously reported to be induced by wounding in loblolly pine (Ruel et al., 1998; Lombardero et al., 2000).

However, the speed and magnitude of induced increases in resin flow suggest that this response may be important in the defense system of spruce (see section 4.8).

4.2. Methyl jasmonate induces increases in terpene content, but only minor changes in terpene composition

Following MJ application, there was a greater accumulation of monoterpenes, sesquiterpenes and diterpenes in *P. abies* sapwood. In all of the various experiments conducted, with trees of 15, 30 or 60 years old, the terpene content of sapwood increased approximately two to three-fold after 100 mM methyl jasmonate application. Unfortunately, the full increase in terpenes following treatment cannot be determined, since we cannot accurately measure the amount of terpenes exuded onto the bark due to losses of polymerization and evaporation during the long sampling intervals used. In several cases, the increases in terpene content were followed later by declines. In the *C. polonica* experiment, the declines in sapwood monoterpene and sesquiterpene content observed for many of the clones at the September sampling point (MJ treatment was carried out in May) may be attributed to outflow of resin from the traumatic ducts in the sapwood to the bark surface or other tissues. Alternative explanations for these declines, such as the volatilization of resin from internal tissues and catabolism of resin terpenes, seem much less likely (Gershenzon, 1994a,b).

In the *I. typographus* experiment, declines were less evident in the wood than in the bark, probably because much of the wood terpenes are stored for a much longer time in the traumatic ducts and/or because the ducts continued to synthesize terpenes; traumatic ducts may remain active for several years (Francheschi et al., 2005).

The 2 to 3-fold increase in terpene content observed in this study was an order of magnitude less than that reported for monoterpenes and diterpenes in the sapwood of two-year-old *Picea abies* saplings after treatment with 10 mM methyl jasmonate (Martin et al., 2002), where foliar application of MJ caused a 10- and 40-fold increase in monoterpenes and diterpenes, respectively. However, in these saplings no resin flow was observed on the bark surface. The amount of resin induced in mature trees may be comparable to that induced in saplings if the bark resin were taken into account. On the other hand, the amount of MJ or JA actually entering the tissues in these experiments might be much lower than the application

concentration. In particular, much higher concentrations of MJ may reach the metabolically active tissues when MJ is sprayed onto the foliage of young plants than when it is applied on the outer bark of older trees (Franceschi et al., 2002). This assumption is supported by the finding that there were no toxic effects, such as tissue browning or degradation in the present work, unlike in the sapling studies, and the effect appeared to be transitory. That is, generally only a single row of traumatic ducts was formed in response to MJ treatment, whereas multiple layers are often formed in response to bark beetle attacks and artificial wounding (Hudgins et al., 2004).

Independent of the treatment, the strongest terpenoid accumulation in both bark and needles of mature trees was recorded ca. four weeks after treatment, a time interval similar to that reported for methyl jasmonate treatments of *Picea abies* saplings (Martin et al., 2002). This suggests that the same mechanism is involved even though the degree of response was different. This time frame is consistent with previous reports of the induction of MJ induced traumatic ducts formation and new resin production in Norway spruce (Nagy et al., 2000; Martin et al., 2002), and after white pine weevil feeding in Sitka spruce (Miller et al., 2005).

Although chemotypes are known to exist for many conifers (Zeneli et al., 2001), trees from different clones and provenances of *P. abies* investigated contained the same basic complement of monoterpenes, sesquiterpenes, and diterpene resin acids. The terpene composition of the resin from mature *Picea abies* stems measured in this study is generally consistent with earlier literature reports on this species concerning the dominance of α-pinene and β-pinene, which alone accounted for ca. 65-70% of the total monoterpenes and the great variability of Δ-3-carene among individuals (Persson et al., 1996; Silvestrini et al., 2004; Sjödin et al., 2000). However, as previously mentioned, the pre-treatment concentrations (constitutive levels) varied between clones. Sesquiterpenes constituted only a minor part of the total terpenes and are represented only by four compounds, namely, α-humulene, longifolene, germacrene and δ-cadinene. The diterpene fraction was dominated by levopimaric acid and abietic acid, accounting for ca. 55-60% of the total diterpenes.

The effect of treatments on the production of terpenes in conifers seems to be quantitative rather than qualitative, in that the response of the tree is characterized by changes in the relative concentrations of most of the bark terpenes (Fig. 3.7 and Fig. 3.9), rather than induction of novel terpenes, and no substantial alteration of the terpene composition of *Picea*

abies resin was observed. Similar results were obtained for *Pinus* species (Heijari et al., 2005). In contrast, terpene composition has been demonstrated to be significantly altered by mechanical wounding, herbivory or pathogen infestation in other conifers, such as *Abies grandis* (Raffa and Berryman, 1987). The terpene composition of species of the genera *Picea* and *Pinus* may be less responsive to environmental stimuli than those of *Abies* because *Picea* and *Pinus* species have larger standing pools of resin stored in constitutive ducts while *Abies* species produce resin in blisters after induction (Lewinsohn et al., 1991a,b). Most previous studies of *Picea abies* and other *Picea* species showed little change in composition on insect or pathogen attack (Lindberg et al., 1992; Martin et al., 2002; Miller et al., 2005) although there are exceptions (Baier et al., 2002).

We found slight differences in constitutive terpenes between bark and needles which is in agreement with previous findings that terpene concentration and composition depend on the tissue (Kleinhentz et al., 1999), time of sampling and geographical location (Zeneli et al., 2001).

The enantiomeric composition of the monoterpene olefins was also generally consistent with previous work (Persson et al., 1996; Sjodin et al., 2000; Silvestrini et al., 2004) except for the ratio of (+) and (-)-α-pinene which is much higher than that in previous reports. This could be partly because of clonal differences and because the content of (-)-α-pinene is very variable (23.6 -51.7%)(Persson et al., 1996). However, no treatment effect on enantiomeric ratios was observed.

4.3. Methyl jasmonate did not alter soluble phenolic content

In *Picea abies*, phenolics are reported to be produced in a certain cell type found in the secondary phloem referred to as polyphenolic parenchyma (PP) cells (Franceschi et al., 1998, 2000, 2002, Nagy et al., 2000). This conclusion is based on the intense fluorescence of these cells under 450-490 nm light (Franceschi et al., 2000) and strong staining with the periodic acid-Schiff procedure, as well as the presence of phenylalanine ammonia lyase (PAL) localized to the PP cells by immunological methods (Franceschi et al., 1998). In analogy with terpenes, one might expect that different species produce different phenolic components, dependent upon the type of organisms commonly attacking them but there is only minor

evidence that relative resistance to pathogens might partly be a function of the type of phenolics produced (Bonello et al., 2003).

The stilbene glycoside composition found in this study is in agreement with those previously described for *Picea abies* by Viiri et al., (2001) and Lindberg et al., (1992). Stilbene aglycones (resveratrol, astringenin and isorhapontigenin) although reported to be present in the bark tissue of Norway spruce (Viiri et al., 2001, Evensen et al., 2000), were not detected in our study or an earlier one (Brignolas et al., 1998), suggesting that they are very variable or that different methods of sample preparation and analysis might influence detection levels. The concentrations of phenolics in *Picea abies* are known to vary according to age, season, provenance and site (Toscano-Underwood and Pearce 1991a,b; Lindberg et al., 1992).

In contrast to the results with terpenes, methyl jasmonate application did not have any significant effects on the levels of soluble phenolic compounds in this study. None of the major stilbenes or flavonoids showed any substantial changes over a period of 4 weeks after treatment, although the PP cells showed similar anatomical changes as after fungal infection. Similarly, Brignolas et al., (1998) found no changes in stilbene aglycones, in fungus-inoculated phloem of Norway spruce. The concentration of total phenolics in lesions after inoculation of red pine (*Pinus resinosa* Ait.) with *Leptographium terebrantis* or mechanical wounding did not significantly differ from the pre-treatment levels (Klepzig et al., 1995).

Perhaps other phenolic compounds that have not yet been measured in *Picea abies* bark are the ones associated with the anatomical changes in the PP cells, such as high molecular weight condensed tannins (Behrens et al., 2003; Maie et al., 2003) or cell wall-bound substances (Strack et al., 1988). These other phenolics may also be responsible for conifer protection against pathogens and bark beetles since the defensive role of the stilbenes and flavonoids and simple phenylpropanoids described to date is ambiguous. *In vitro* tests showed that these compounds have antifungal properties against certain pathogens (Shain, 1971; Woodward and Pearce, 1988a,b), but not against others (Evensen et al., 2000), and they did not affect bark beetle feeding (McNee et al., 2003). Moreover, crude methanol extracts (which would be expected to contain nearly all stilbenes, flavonoids and simple phenolic conjugates present) exhibited little or no inhibition of fungal growth (Evensen et al., 2000). Perhaps the situation *in vitro* is different due to the presence of activating enzymes or other factors. To

clarify the importance of phenolics in conifer defense, attempts must be made to carry out more extensive phenolic analyses as well as to manipulate phenolic levels *in vivo*.

4.4. Induced terpene defences and resistance to *Ceratocystic polonica*

Methyl jasmonate treatment not only increased terpene content and resin flow, but also increased resistance to the fungus *Ceratocystis polonica*. The growth of this blue-staining fungus into the sapwood and the necrosis of the cambium caused by fungal invasion were both significantly reduced by methyl jasmonate application (Fig. 3.12). In previous investigations of *Picea abies*, similar reductions of *Ceratocystis polonica* infestation were achieved by prior inoculation with a sublethal dose of *Ceratocystis polonica* (Krokene et al., 1999, 2001, 2003), a treatment that also may have acted by increasing the terpene content. In this study, the correlation between terpene content and fungal resistance is especially striking when one compares the different clonal lines investigated. While the five clones that showed a significant increase in terpene content after jasmonate application also showed significant declines in fungal growth, clone 63 showed no increase in terpenes and at the same time was very susceptible to fungal invasion. Terpene enrichment near an area of wounding is typical of other conifers, especially in response to pathogen inoculation (Raffa and Berryman, 1982; Croteau et al., 1987; Werner and Illman, 1994; Klepzig et al., 1995). It seems that MJ had the same effect on triggering defense responses against *Ceratocystis polonica* as previously shown to protect Norway spruce seedlings against *Pythium ultimum* Trow. (Kozlowski et al., 1999). The correlation between terpene content and fungal resistance is also strengthened by data showing that trees that survived the *Ceratocystis polonica* inoculation had much higher terpene concentrations than trees killed by the fungus.

The ability of *P. abies* to defend itself against *C. polonica* should be closely related to its ability to defend itself against the bark beetle, *I. typographus*, which vectors *C. polonica* as a fungal symbiont. First of all, dose–response experiments comparing fungal inoculations and induced bark beetle attacks implicate pathogenic fungi in tree killing by showing that a similar number of inoculations and barka beetle attacks are required to kill a tree (Christiansen, 1985a,b). *C. polonica* is shown to be at the leading edge of fungal penetration all the way to the heartwood in Norway spruce attacked by *I. typographus* (Solheim, 1992), producing more

and deeper stain/desiccation further into the sapwood than the other fungi (Krokene and Solheim, 1998). Moreover, the fact that all aggressive bark beetles, such as *I. typographus*, are associated with relatively virulent blue-stain fungi, and that these fungi usually are relatively vector-specific, also suggests that blue-stain fungi may be important in tree killing (Franceschi et al., 2005). Even if one believes that the beetle-fungus complex probably acts synergistically to overwhelm host defenses, rather than the fungus alone, again it is critical for the beetle how the fungus will perform. Thus, a decline in the fungus growth declines will also affect beetle attack success. Since most individuals of *I. typographus* carry *C. polonica* (Krokene and Solheim, 1996), an effective *P. abies* defense against the beetle must almost always involve defense against the fungus.

4.5. Induced terpene defences and resistance to bark beetles

MJ application to mature Norway spruce growing in a wild stand also induced resistance against bark beetle attack. Bark sections of *Picea abies* treated with MJ had significantly less *I typographus* colonization than control bark, with shorter parental galleries excavated and fewer eggs deposited. The numbers of beetles that emerged and mean dry weight per beetle were also significantly lower in MJ-treated bark. In addition, fewer beetles were attracted to conspecifics tunneling in MJ-treated bark. These effects can be discussed in relation to the host colonization sequence of the beetles, from selection to concentration, establishment and dispersal, as diagrammed in Figure 4.1 (D.L. Wood, 1982). The selection phase begins when the beetles respond to host stimuli before or after landing on the bark, and ends with sustained feeding in the phloem. Primary host selection in bark beetles has been suggested to be either a random process, in which beetles land on trees without any external stimuli (Moeck et al., 1981; Byers, 1995), or a directed response to various chemical and visual stimuli (Byers et al., 1988; Borden, 1989). Among the chemical stimuli, monoterpenes and their enantiomers play and important role in the communication system of the spruce bark beetles and host selection (Persson et al., 1996). *I typographus* produces (+)-*cis*-verbenol and (+)-*trans*-verbenol by oxygenation of the monoterpene precursors (+)-α-pinene and (-)-α -pinene, respectively, obtained from the Norway spruce (*Picea abies*) host (Klimetzek and Francke, 1980; Kohnle et al., 1988).

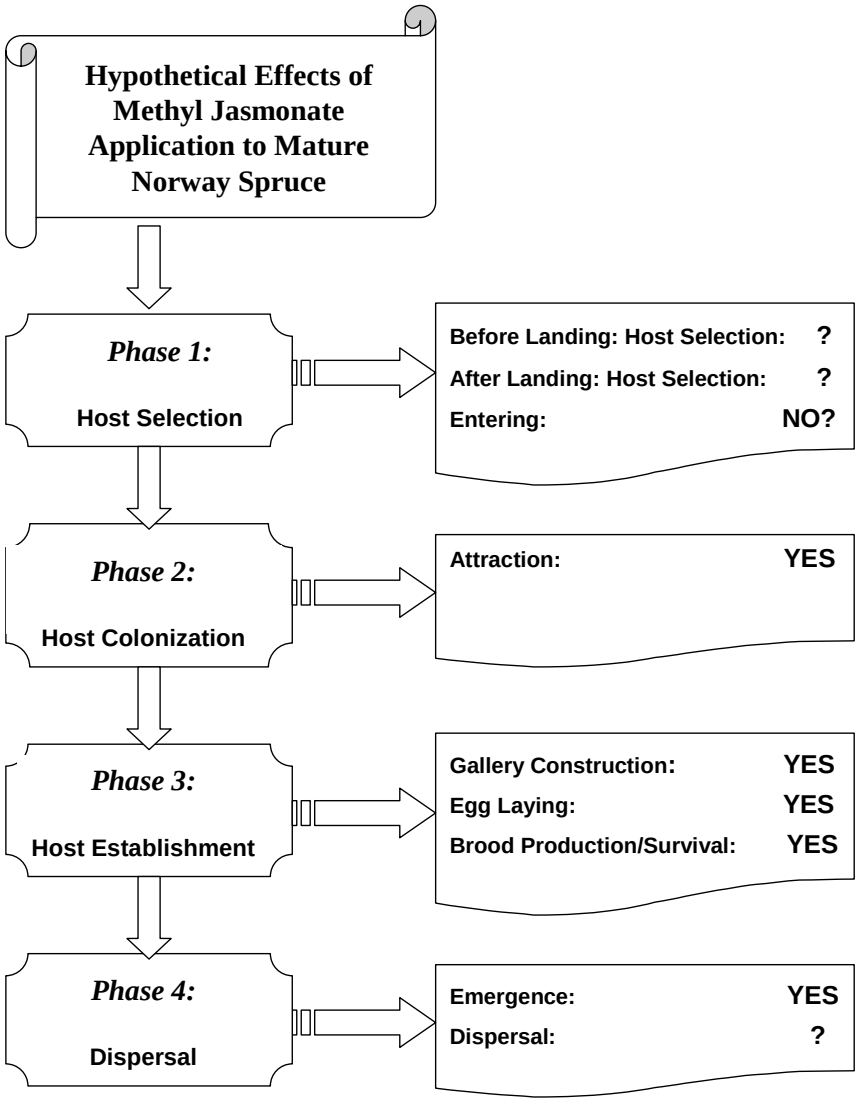

Figure 4.1. Conceptual diagram of the different stages in *I. typographus* host selection and development with an indication of whether (YES) or not (NO) methyl jasmonate treatment had an effect on each stage. See text for further details.

MJ treatment did not appear to have strong effects on the beetle's decision to enter the bark, and hence any influence of MJ on host selection before or shortly after landing on the host is also likely to be small.

However, the absence of clear effects of MJ on beetle entry could be due to our use of synthetic aggregation pheromones to initiate attacks on the trees. The pheromone emission from the dispensers corresponds to about 200 pheromone-producing males (Birgersson and Bergström, 1989), and this signal might override any deterring effects of MJ treatment and so

increase the propensity of the beetles to enter unsuitable hosts (Borden, 1989; Camacho et al., 1994). However, since MJ also did not affect entry behaviour in the bark disc assay where no pheromones were used this possibility seems unlikely. Although we did not obtain evidence for an effect of MJ on so called primary (or host-induced) attraction of "pioneer beetles" (i.e. the first beetles to arrive on a tree), the total emission of volatile terpenes has been shown to increase 5-fold after foliar application of MJ on two year-old saplings, with individual compounds such as (*E*)-β-farnesene and linalool increasing up to 100-fold (Martin et al., 2003). These compounds are of particular ecological interest, as they have been reported to attract natural enemies of herbivores or repel herbivores directly in other plant species (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002). The possibility that similar effects occur in the Norway spruce – *I typographus* system deserves further investigation.

The concentration phase begins when flying beetles respond to aggregation pheromones and/or host compounds (secondary attraction), continues with production of aggregation pheromones, and ends with the production of antiaggregation pheromones (D.L. Wood, 1982). This process is crucial for the survival of the dispersing population of tree-killing bark beetles since they rely on mass attacks of host trees to obtain substrate for larval development. MJ appeared to interfere with pheromone production/communication of *I typographus*, since fewer beetles were attracted to beetles tunneling in MJ-treated bark as compared to beetles tunneling in untreated bark. One possible explanation is that MJ treatment reduces the level of precursors for pheromone production. Indeed, pheromone production seems to be proportional to the extent of beetle tunneling, since there was a strong correlation between bark beetle attraction and gallery length. Similar results have been reported for the congeneric species *I pini* Say (Erbilgin and Raffa, 2000). However, since *I typographus* and other *I* species can synthesize at least some pheromone components *de novo* without using host precursors (Lanne et al., 1989; Seybold et al., 1995), it is also possible that tree defenses induced by MJ interfere with pheromone emission. Raffa and Berryman (1983) found for example that *Dendroctonus ponderosa* Hopkins beetles that were continuously fighting resin defenses in vigorous trees were unable to initiate mass attacks. Additional studies are necessary to understand how MJ interferes with pheromone production and communication in bark beetles.

MJ strongly inhibited the establishment phase of host colonization, which begins when the mortality of the host tree is assured. This phase starts with the initiation of mating, gallery

construction and oviposition and ends when the elongation of egg galleries and oviposition cease. MJ treatment strongly influenced gallery construction and egg laying by *I typographus*. Even though the total number of attacks was similar on both sides of the trees, incipient gallery construction was much less extensive on the MJ treated side, indicating that these tissues were less suitable for the beetles.

Another plausible explanation is that chemical changes in the phloem affected the ability of bark beetles to excavate host tissues and lay eggs. Currently we do not know if *I typographus* is less tolerant to increased quantities of terpenes, but studies on *I pini* indicate that tunneling in a phloem-based medium decreases with increasing concentration of α-pinene in the medium (Wallin and Raffa, 2000). The phytopathogenic fungal associates of the beetles play a crucial role during the establishment phase by helping to overcome tree resistance (Paine et al., 1997). Earlier studies have shown that MJ treatment increases resistance to *Ceratocystis polonica* and other pathogens in Norway spruce (Kozlowski et al., 1999; Franceschi et al., 2002; Schmidt et al., 2005). Thus MJ treatment might well have affected beetle colonization via its effect on fungal associates.

MJ application also reduced both the quality and quantity of brood production by *I typographus*. These results agrees with studies showing that bark beetles colonizing trees whose defenses had been induced usually experienced increased levels of egg and larval mortality (Nebeker et al., 1993; Raffa and Smalley, 1995). For example, *I typographus* was less successful in attacking Norway spruce that had been elicited by a sub-lethal number of inoculations with the fungus *Ceratocystis polonica* (Christiansen and Krokene, 1999).

The dispersal phase includes offspring emergence from natal trees and response to host stimuli and/or aggregation pheromones. MJ application is likely to have negatively influenced the fitness and dispersal ability of emerging bark beetles by reducing their body weight and lipid content. Although, we did not determine lipid content directly, body weight and lipid content are directly correlated in *I typographus* (Anderbrant et al., 1985). Lipids are used as an energy source during dispersal (Slansky and Haack, 1986) and has been shown to affect bark beetle survival, host colonization, host selection and reproduction (Anderbrant et al., 1985; Anderbrant, 1988; Wallin and Raffa, 2004).

4.6. Induced defenses and resistance to white pine weevils feeding

In addition to studying an insect herbivore of the trunk and its fungal symbiont, we also included in our research a shoot infesting insect. Unlike bark beetles that feed on trees of at least 50 years old, white pine weevils feed on 1-2 year old tissue (Alfaro et al., 2002). In contrast to our other experiments, where increase in terpenes was correlated with increase in tree resistance against bark beetle and its associated fungi, the applied treatments had no impact on the white pine weevil biological performances. Neither JA injection nor wounding treatments had an effect on white pine weevil feeding activities.

These differences in response might be because of several reasons. First, the method of jasmonate application was different (painting *vs* jasmonic acid injections). Terpenes are known to accumulate after weevil attack (Miller et al., 2005) and in drill-wounded white spruce stems (Tomlin et al., 2000). In these studies, changes in volatile terpene and diterpene resin acid content are much higher than those found in the present study. Perhaps the dose of jasmonic acid absorbed was not sufficient for induction or this compound was not transported well to the site of weevil attack.

Secondly, perhaps *P. strobi* is more resistant to the terpenes of its host than the other pests that we studied. Herbivores confronted with plant defenses may evolve to cope with them (Ehrlich and Raven, 1964). Tolerance to monoterpenes, the most conspicuous and well-studied constituents of conifer resin, has been suggested to be characteristic for aggressive bark beetle species (Berryman et al., 1989). A similar phenomenon has been reported for the old house borer, *Hylotrupes bajulus* (Nerg et al., 2004). It has been shown that total monoterpene concentration in wood was positively associated with mean relative growth rate of larvae and oviposition of adults, and thus it was postulated that individual terpenoid compounds are more important in determining the performance of wood-boring insects rather than total amounts (Nerg et al., 2004). Some bark beetle species are also tolerant to conifer monoterpenes, with the most tolerant species those that breed solitarily in living trees and those that colonize tree species with well-developed constitutive resin defenses (Raffa, 1991). Some degree of resin tolerance is probably common also in shoot infesting insect, because even dying and dead leaders contain residual resin. A low level of tolerance to host terpenoid resins may thus be widespread among weevils.

Another goal of the work on *P. strobi* was to compare changes in terpene content caused by this insect with that caused by mechanical damage and JA. We found several differences in the responses of the bark and needles between the insect and jasmonic acid treatments. In the bark tissue, feeding of the weevil induced an increase in monoterpenoid and sesquiterpenoid content, but caused no significant changes in the proportion of the different diterpenes in the oleoresin or other compositional changes. Similarly, Kleinhentz et al., (1999) found no qualitative difference between maritime pine trees that were attacked by the European stem borer, *Dioryctria sylvestrella*, compared with trees that were not attacked. Miller et al., (2005) also reported that the major terpenes in the reaction lesion of Sitka spruce trees in response to white pine weevil infestation did not differ from the constitutive resin, and that only minor compositional changes occurred in both their inner and outer stems.

4.7. Induced defense: comparing spruce and herbaceous plants

Based on our current knowledge, the chemistry of induced defenses in *Picea abies* and other woody plants is not materially different from that of herbaceous plants. Terpenes and phenolics are all common metabolites in herbs, and in many cases are inducible upon herbivory or pathogen infection (Sabelis et al., 1999; Wittstock and Gershenzon, 2002; Degenhardt et al., 2003; Hammerschmidt, 2005). However, the presence of inducible terpene resins is a special feature of conifers. In the rest of the plant kingdom, mixtures of terpenes accumulate in resin ducts, cavities or glandular hairs of many taxa, but are usually not reported to be inducible (Gershenzon and Croteau, 1991). Moreover, the long induction time of conifer resin also sets it apart from other induced defenses.

The occurrence of multiple defense systems in a single plant species is also not unique to gymnosperms or any other plant group. It has been suggested that different defense systems target different types of pests (Paine et al., 1997). However, terpene resins, based on their bioassay results and physical properties, could act as barriers against both, herbivores or pathogens (Phillips and Croteau, 1999; Trapp and Croteau, 2001). Indeed, attack on *Picea abies* by an herbivore such as *I typographus*, is often accompanied by infestation of fungi dispersed by the herbivore, such as *C. polonica*. Thus, the possession of defenses active against multiple enemies is easy to explain.

The regulation of induced defenses in spruce and angiosperms is also similar as far as the role of jasmonates are concerned. However, the concentrations of methyl jasmonate found to be effective in spraying *Picea abies* trees in our work (maximum effect at 10 or 100 mM) were relatively high compared to those typically used on angiosperm foliage: 10 μM – 1 mM (Dicke et al., 1999; Thaler, 1999; Thaler et al., 2002). This may only be a consequence of the need for higher concentrations to penetrate the thick bark of large Norway spruce trees.

4.8. Cost and induced defense

Norway spruce trees are challenged by a wide variety of enemies during their long lives. Among those causing the most damage are the three species investigated in this thesis project, the bark beetle, *I. typographus*, its associated fungus, *C. polonica*, and the weevil, *P. strobi*. Although defenses against such enemies may be costly, the potential severe consequences of herbivory and disease for tree mortality and reproductive output, particularly for long-lived species, such as *P. abies*, that do not reproduce for many years (Skrøppa, 2003), have selected for significant amounts of constitutive and induced defenses. Inducible defenses are considered to be less costly than constitutive defenses because they are only produced following initial attack (Karban and Baldwin, 1997) and can be fine-tuned based on the severity of the attack (Baldwin, 1998). However, it is not easy to assess how such costs affect fitness in long-lived trees, such as *P. abies*, that reproduce over many years and may have large pools of reserve carbohydrate and nutrients. Nevertheless, costs can be clearly seen for the production of traumatic ducts in *P. abies* because the tree foregoes xylem formation to make these new structures, thus reducing transport capabilities. We have not yet looked for declines in growth that may accompany traumatic ducts formation, although there is evidence for these in few gymnosperms (Wainhouse et al., 1998; Baier et al., 2002; Heijari et al., 2005).

To be effective, induced defenses must be made rapidly enough to significantly reduce herbivore or pathogen damage. The battle between a plant and an invading pathogen is often dependent on speed. The winner is determined by how quickly the pathogen can proliferate and exert damage, compared to how fast the plant can respond with the necessary levels of defense. Thus, timing, rather than the magnitude of the response, is often the critical factor in host-pathogen interactions (Luchi et al., 2005). Is the timing of induced defense production

observed in this study rapid enough to protect *P. abies* from its enemies? The increase in terpene content following methyl jasmonate application was first detected after four weeks in the multiple clone experiment (Fig. 3.8). In the single clone experiment it occurred between the 8 and 35 day sampling points (Fig. 3.9). The timeframe of terpenoid-based responses was also similar in the *I. typographus* experiments, and correspondingly, *P. abies* were found to be resistant to subsequent *C. polonica* inoculation and bark beetle attack 4 weeks after methyl jasmonate application. In the *P. strobi* experiments, the timing of defense induction after jasmonic acid injection and mechanical wounding was also similar. Thus, induced terpene defenses in *P. abies* require at least 2-4 weeks for deployment (Krekling et al., 2004).

Under natural conditions, when *I. typographus* attacks *P. abies* and *C. polonica* is inoculated, the pace of events can be much faster than the formation of traumatic ducts. Bark beetles, such as *I. typographus*, may mass attack and successfully colonize mature *Picea abies* within a week (Christiansen and Bakke, 1988; Franceschi et al., 2000; Wermelinger, 2004). The same holds true in case of white pine weevil attack (Alfaro, 1994; 1998; Boucher et al, 2001b). In such cases, the increase in terpene content arising from traumatic duct formation after initial invasion would seem to be too slow to have defensive value, unless insect attack is interrupted by adverse flight conditions. However, during a typical Scandinavian summer, this may indeed be the case; favorable weather conditions may not last long enough to allow for these rapid attacks (Christiansen, 1985b; Økland and Bjørnstad, 2003; Rolland and Lempériére, 2004; Wermelinger, 2004), and so induced resin may form an effective defense against *I. typographus* and its fungal symbiont. Even if induced terpene defenses are too slow to stop initial attacks, they still could have value if initial attacks are not lethal due to constitutive defenses. In such cases, induced defenses may be formed in preparation for later attacks. Initial attack is likely to be a good indicator of additional attacks, especially when bark beetle populations are high.

4.9. Terpene variability, a rule rather than an exception in *Picea abies*

The induced terpene defenses of *P. abies* formed following jasmonate induction showed complexity and variability at several levels of organization. First, the induced resin contains a mixture of 22 monoterpenes, sesquiterpenes and diterpenes at the level of detection employed.

Second, there was variation in terpene composition and total content among the organs of single tree (needles *vs* bark), among different trees of a single clone, and among different clones.

Such differences have been often reported for terpenes in other conifer species. For example, the xylem, bark and needles of blue spruce (*Picea pungens* Englem) produce different terpenes (Moore and Hanover, 1987). The same phenomenon is reported for the amount of terpenes in the needles vs. twigs of Balkan pine (*Pinus peuce* Grisebach) (Papadopoulou and Koukos, 1996). High variability is also reported to be present in needle and wood terpenoids in Scots pine provenances (Manninen et al., 2002), silver fir (Zeneli et al., 2001) and the species studied here, *P. abies* (Persson et al., 1996).

The patterns of complexity and variability of induced terpenes in spruce and other conifers might have several ecological consequences for their role as a defensive trait. In complex mixtures, different types of terpenes may target specific enemies or may synergize each other's actions (Cates, 1996; Michelozzi, 1999). For example, the monoterpene hydrocarbons of *P. abies* are very non-polar and may promote the ability of other terpenes to penetrate membranes and reach their site of action. Complex mixtures may also confound the capacity of herbivores to evolve resistance, and hence slow the rate of breakthrough of a plant defense (Langenheim, 1994). Maintaining resistance in evolutionary time is a challenge for a long-lived plant, such as spruce whose insect herbivores and pathogens have much shorter generation times (Hansson, 2003). The large terpene mixtures present in *P. abies* also give an opportunity for variability to be present among organs, developmental stages and individuals. Such variability itself is a useful defense because it may restrict damage caused by herbivores or pathogens that evolve resistance to one terpenoid profile (Barnola et al., 1997; Cates, 1996; Mita el al, 2002) and attackers may take much longer to find suitable hosts (Barnola et al., 1997). There may be value in simply being different. Plants whose terpene composition differs from that of the average have been suggested to be more resistant to adapted herbivores (Langenheim, 1994).

4.10. Regulation of terpene formation in *Picea abies* cell cultures

P. abies cell suspension cultures constitutively accumulated small amounts of various hydrocarbon monoterpenes with a product profile similar to that of adult trees. This contrasts with previous reports in other conifers, where no monoterpenes were found in cell suspensions of lodgepole pine (*Pinus contorta),* slash pine (*Pinus eliotii*), or Douglas-fir (*Pseudotsuga menziesii*) (Lewinsohn et at., 1994), or in previous work with *P. abies* suspension cultures (Lindmark-Henriksson et al., 2003, 2004). The difference could be attributed to good luck in the choice of cell lines, the use of different elicitors at different times in the cell cycle, or the more sensitive monoterpene detection method employed, a hydrophobic resin (XAD-4) added to cultures rather than simple solvent extraction. Monoterpenes have high volatiles at room temperature and the resin may have helped trap the small amounts produced before they are given off or are catabolized (Falk et al., 1990).

The accumulation of monoterpenes in *P. abies* cultures allowed us to investigate how their formation is regulated. Both chitosan (a component of fungal cell walls) and MJ induced greater monoterpene accumulation. The effect of chitosan is especially striking and indicates that the fungus itself is responsible for triggering monoterpene formation after bark beetle attack on mature trees. The increases in monoterpenes after chitosan and MJ application, first observed 72 h after elicitation, was preceded by a rise in monoterpene synthase activity. These results suggest that greater accumulation was due to an increased rate of biosynthesis, rather than changes in the rate of catabolism.

Comparison of the monoterpene composition of elicited and nonelicited cultures indicates that elicitation does not result in the production of new types of monoterpenes, but simply affects the abundance of existing ones. The same phenomenon was reported by Ketchum et al., (1999) in case of taxoid accumulation. In contrary to Yukimune et al., (1996) who described a 4-day lag phase in the paclitaxel (a diterpenoid) accumulation from *Taxus* cultures, following elicitation, no lag phase was detected in our cultures, possibly because methyl jasmonate was added to a 3-day-old culture, rather than to newly sub-cultured cells.

Monoterpene synthase activity was significantly induced by both chitosan and MJ elicitors. The time-course of the change in total monoterpene synthase activity after elicitor induction showed a steady increase in activity to about 72 hr and then a slow decline. This

increase in synthase activity was observed on both a protein and gram fresh weight basis. The rapid reaction of spruce cell suspension cultures is similar to elicitor-induced responses in other plant cell systems (Lewinsohn et at., 1994). This contrasts markedly with the relatively slow MJ-elicited response in intact *P. abies* saplings in which maximum induction of monoterpene synthase activity occurred after about 10 days (Martin et al., 2002). This difference may be a consequence of the longer time required for the transport of MJ after application from outer bark to the monoterpene-synthesizing tissue, or the fact that brand new monoterpene-producing cells must first be formed in intact tissue before accumulation can occur (Martin et al., 2002). Similarly, a much slower response to elicitor challenge was noted in grand fir stems (Steele et al., 1998a, 1998b), as compared to cell suspensions cultures of the same species (Lewinsohn et at., 1994). Although the response in spruce cell suspension cultures was much faster than the response after MJ application in stem tissue (Martin et al., 2002), the levels of induced monoterpene synthase activity in suspension cultures were lower than those observed in wounded stems by a factor of about 10 on either a protein or fresh weight basis. Also, the decline of the monoterpene synthase activity was faster in suspension cultures than observed in wounded stems (96 hours *vs* 18 days).

Comparing the two elicitors, monoterpene synthase is the most significantly activated after MJ application, in contrast with findings of Zhao et al., (2006) who found that monoterpene synthase activity from yeast elicitor-treated *Cupressus lusitanica* cell cultures was higher than from MJ-elicited cultures. Perhaps the mode of action of the yeast elicitor is different from that of the chitosan used in our study. The monoterpene synthase activity increased 20 minutes after elicitation, being more than 2-fold over the control, and reached more than 3-fold over the control after 12 hours. This response is similar to wounding-induced grand fir and yeast elicitor-treated *Cupressus lusitanica* cell cultures monoterpene synthases (Steele et al., 1998b; Zhao et al., 2005).

The *in vitro* assays suggested that only monoterpene synthases were induced, not sesquiterpene or diterpene synthases. This fits with the lack of sesquiterpene and diterpene accumulation in culture. The major reason for lack of accumulation of these higher terpenes might be the lack of specialized storage structures. Moreover, diterpene resin acids are thought to be toxic at high concentration (Trap and Croteau, 2001).

The patterns of monoterpene production and the activity of the monoterpene synthase enzymes are similar following application of both the fungal elicitor and MJ, suggesting that the elicitor acts by triggering the jasmonate signaling pathway, consistent with the dramatic effect of MJ on terpene accumulation described elsewhere in this thesis. Jasmonates serve as key signaling compounds for the activation of complex defense changes in multiple conifer lineages (Hudgins et al., 2004). However, there is only one previous report of endogenous jasmonates in a conifer, *Taxus* (Mueller et al., 1993), an observation made following elicitor and pathogen treatments. The nature of the signal cascade between the fungus and jasmonate, on the one hand, and jasmonate and monoterpene biosynthesis, on the other, is still unknown. Other signaling molecules, such as ethylene and reactive oxygen species, may be involved. However, recent advances in the biochemistry and molecular biology of conifer defenses now bode well for progress on defense signaling.

SUMMARY

The terpenoid resin and phenolic constituents of conifers have been implicated in protecting trees against bark beetles and other enemies. This implication has been based on their toxicity and repellency *in vitro*, their induction around the point of attack and, in the case of terpene resins, their physical properties and mode of storage. However, the results of *in vitro* bioassays have sometimes been equivocal because it is very hard to simulate the physical arrangements of the living plant (e.g., the exudation of resin) *in vitro*. It would be best to test defensive roles *in vivo* in intact plants. *In vivo* experiments require a method of manipulating defense level with minimal effects on other aspects of plant phenotypes. Methyl jasmonate, a well-known inducer of plant defense responses, was used to manipulate the biochemistry and anatomy of mature *Picea abies* (Norway spruce) stems. We determined the effect of methyl jasmonate on terpenoids and other chemical defenses of mature *Picea abies*, and investigated if this treatment protected trees against attack by the blue-stain fungus *Ceratocystis polonica* [(Siem.) C. Moreau], the spruce bark beetle (*Ips typographus* L.) and the white pine weevil (*Pissodes strobi* Peck.).

Methyl jasmonate treatment induced the formation of a new row of resin ducts in the sapwood (the newly developing xylem), which was also associated with a significant increase in resin accumulation and flow onto the outer bark. MJ treatment stimulated increased accumulation of all the major terpene classes in resin, including monoterpenes, sesquiterpenes, and diterpene resin acids. The total terpene content of sapwood increased approximately two to three-fold after 100 mM methyl jasmonate application. Only minor changes were detected in terpene composition, including enantiomeric composition. These results were consistent across all experiments and clonal lines used, but there was a high variability among clones in the timing and degree of response to methyl jasmonate.

Contrary to its effect on terpenes, methyl jasmonate application did not have any significant effects on the levels of soluble phenolic compounds. None of the major stilbenes or flavonoids showed any substantial changes over a period of 4 weeks after treatment, although the phloem parenchyma cells that are said to produce phenolics showed dramatic anatomical changes similar to those occurring after fungal infection.

The observed chemical and anatomical changes were correlated with increased resistance to the spruce bark beetle, *I. typographus*, and its fungal associate, *C. polonica*, thought to be an

important cause of bark beetle-caused death of *P. abies*. The growth of *C. polonica*, a blue-staining fungus, into the sapwood and the necrosis of the cambium caused by fungal invasion were both significantly reduced by methyl jasmonate application, suggesting that terpenoid oleoresin may function in defense against this pathogen. The correlation between terpene content and fungal resistance is also strengthened by data showing that trees that survived the *Ceratocystis polonica* inoculation had much higher terpene concentrations than trees killed by the fungus.

In experiments to study resistance to the spruce bark beetle, *I. typographus*, bark sections of *P. abies* treated with methyl jasmonate had significantly less *I. typographus* colonization than control bark, with shorter parental galleries excavated and fewer eggs deposited. The numbers of beetles that emerged and mean dry weight per beetle were also significantly lower in methyl jasmonate-treated bark. In these experiments, fewer beetles were attracted to conspecifics tunneling in methyl jasmonate-treated bark. *P. abies* stem sections treated with methyl jasmonate again had an increased number of traumatic resin ducts and a higher concentration of terpenes than untreated sections, whereas the concentration of soluble phenolics did not differ between treatments. Thus, the increased amount of terpenoid resin present in methyl jasmonate-treated bark could be directly responsible for the observed decrease in *I. typographus* colonization and reproduction.

To study resistance to the white pine weevil, *Pissodes strobi,* jasmonic acid was injected into the terminal stems of *P. abies*, the feeding site of this insect. However, this treatment had no effect on *P. strobi* performance. Nevertheless, jasmonic acid, as well as weevil infection and mechanical wounding did cause changes in the terpene content of terminal stems. In the bark tissue, feeding by the white pine weevil and mechanical wounding, but not jasmonic acid, induced a near 2 fold increase in monoterpenoid and sesquiterpenoid content, but caused no significant changes in the proportion of the different diterpenes in the oleoresin. In the needles, both weevil feeding and jasmonic acid, but not mechanical wounding, caused an increase of almost 2-fold in monoterpene and sesquiterpene accumulation and 3-fold in diterpene accumulation 32 days after the treatments began. Thus, although weevil feeding altered terpene content, these changes had no effect on resistance under the conditions tested.

To study the role of jasmonate-dependent signaling pathways in the formation of induced defenses in *P. abies*, we took advantage of the fact that cell cultures offer an experimentally more tractable and well-defined system for examining this localized defense response than mature, fully-differentiated trees. *P. abies* cell suspension cultures constitutively biosynthesized small amounts of various monoterpene hydrocarbons *de novo* with a product profile similar to that of adult trees. No accumulation of sesquiterpenes or diterpenes was observed. However, following application of MJ or a fungal elicitor, there was a three-fold increase in monoterpene accumulation. Measurements of monoterpene synthase activity, the committed step in monoterpene biosynthesis, showed that this enzyme activity was significantly induced by both fungal elicitor and MJ, with MJ having the greatest effect. These results confirm that the jasmonate signaling pathway is an important endogenous regulator of induced terpene biosynthesis in spruce.

Taken together, the results of this study suggest that induced defenses such as formation of traumatic resin ducts, enhancement of resin flow, and increased accumulation of volatile terpenes and diterpene resin acids, all triggered by application of jasmonates, play a significant role in Norway spruce defense against multiple enemies. The results also confirmed that the jasmonate pathway directly regulates the formation of these induced defenses.

Further research is necessary to demonstrate the actual defensive roles of these metabolites since the results reported here are all correlative. We cannot exclude that the resistance caused by jasmonate treatment could be a result of defenses other than the terpenes. The specificity of jasmonates to one or a few classes of defense compounds has not yet been demonstrated in conifers. Increasing knowledge of the biochemistry and molecular biology of defense metabolism is now making it easier to identify genes regulating the formation of a single defensive substance or class of defensive compounds in conifers. Such genes can be used to prepare transgenic conifers with altered defense profiles, which should provide ideal material to further test defensive roles of terpenes.

Keywords: bark beetles, cell suspension culture, *Ceratocystis polonica*, conifer, defense responses, elicitor, host colonization sequence, inducible defenses, *Ips typographus*, mechanical wounding, methyl jasmonate, monoterpene, octadecanoid pathway, phenolics, phloem, *Pissodes strobi*, spruce bark beetle, traumatic resin ducts, white pine weevil.

ZUSAMMENFASUNG

Terpenoidharze und phenolische Komponenten sind in Koniferen an Abwehrreaktionen gegen Borkenkäfer und andere Feinde beteiligt. Die repellente Funktion dieser Baumharze, die nach Befall vermehrt aus der Inokulationsstelle des Baumes austreten, beruht auf deren Toxizität *in vitro*, und im Fall von Terpenharzen, auf deren physikalischen Eigenschaften und der speziellen Speicherungsart im Gewebe. Die Ergebnisse von *in vitro*-Bioassays waren jedoch manchmal doppeldeutig, weil es sehr schwer ist, die physischen Gegebenheiten der lebenden Pflanze (z.B. die Absonderung von Harz) *in vitro* zu simulieren. Es wäre besser, die Verteidigungsrollen *in vivo* an intakten Pflanzen zu untersuchen. *In vivo* Experimente erfordern eine Methode zur Manipulierung des Verteidigungsgrades mit geringen Einflüssen auf andere Aspekte von Pflanzenphenotypen.

Methyljasmonat, ein bekannter Induktor von Pflanzenabwehrreaktionen, wurde genutzt, um die Biochemie und Anatomie von ausgewachsenen *Picea abies*-Stämmen (Norwegische Fichte) zu manipulieren. Hierbei wurde die Wirkung von Methyljasmonat auf Terpenoide und andere chemische Verteidigungen von ausgewachsenen *P. abies* bestimmt. Desweiteren wurde untersucht, ob diese Behandlung Bäume gegen den Befall von Blaufäulepilz *Ceratocystis polonica* [(Siem.) C. Moreau], Fichtenborkenkäfer (*Ips typographus* L.) und weißem Kieferrüsselkäfer (*Pissodes strobi* Peck.) schützt.

Methyljasmonatbehandlung führt zur Bildung einer neuen Reihe von Harzgängen im Splintholz (dem sich neu entwickelnden Xylem), das auch mit einer bedeutenden Erhöhung von Harzansammlungen und Harzfluß auf die Außenborke in Verbindung steht. Methyljasmonatbehandlung stimulierte eine erhöhte Ansammlung aller großen Terpenklassen im Harz, einschließlich Monoterpenen, Sesquiterpenen und Diterpenharzsäuren. Der gesamte Terpengehalt von Splintholz stieg nach Behandlung mit 100 mM Methyljasmonat um etwa das Zwei- bis Dreifache. In der Terpenzusammensetzung, einschließlich der Enantiomer-Zusammensetzung, wurden nur geringe Veränderungen gefunden. Diese Ergebnisse waren in allen durchgeführten Experimenten und Klonlinien gleich, allerdings gab es große Schwankungen in den Klonen bezüglich Zeitpunkt und Reaktionsgrad auf Methyljasmonat.

Entgegen seiner Wirkung auf Terpene hatte der Einsatz von Methyljasmonat keine bedeutenden Auswirkungen auf den Grad der löslichen Phenolverbindungen. Keine der größeren Stilbene oder Flavonoide zeigte wesentliche Veränderungen über eine Zeitspanne von 4 Wochen nach Behandlung, obwohl die Phloemparenchymazellen, die eigentlich phenolische Verbindungen wie Phenol produzieren, drastische anatomische Veränderungen, ähnlich jenen, die nach Pilzbefall auftreten, zeigten.

Die beobachteten chemischen und anatomischen Veränderungen wurden mit der erhöhten Resistenz auf Fichtenborkenkäfer, *I. typographus*, und deren Begleitpilz, *C. polonica*, von dem man annahm, dass er ein bedeutender Verursacher des Sterbens von *Picea abies* durch Borkenkäfer ist, in Beziehung gesetzt. In diesem Zusammenhang wurde gezeigt, dass nach Methyljasmonatanwendung das Wachstum sowie die Größe des nekrotischen Kambiums um die Inokulationsstelle des pathogenen Pilzes *C. polonica* im Splintholz bedeutend eingeschränkt ist. Dies führt zu der Annahme, dass Terpenoidoleoharz eine Funktion bei der Verteidigung gegen diese Pathogene besitzen können.

Die Beziehung zwischen Terpengehalt und Pilzabwehr wird ebenfalls durch Daten untermauert, die zeigen, dass Bäume, die die *C. polonica*-Inokulation überlebt haben, eine viel größere Terpenkonzentration aufwiesen als jene, die durch den Pilz abgetötet wurden.

Untersuchungen zur Resistenz gegenüber dem Borkenkäfer, *I. typographus*, zeigten, dass Teile der mit Methyljasmonat behandelten Borken von *P. abies* einen deutlich geringeren *I. typographus*-Befall mit weniger ausgeprägten Fraßgängen und geringerer Eiablage als die nicht behandelten Borken aufwiesen. Die Anzahl der geschlüpften Borkenkäfer und das durchschnittliche Trockengewicht pro Käfer sind signifikant niedriger in mit Methyljasmonat behandelter Borke. In diesen Experimenten sind weniger Käfer in mit Methyljasmonat behandelter Borke zu der arttypischen Durchhöhlung der Borken angeregt worden. Die wiederholte Behandlung von *P. abies*-Stammabschnitten mit Methyljasmonat führte zu einer Vermehrung der Wundharzgänge und einer höheren Konzentration an Terpen als in den unbehandelten Abschnitten, wobei sich die Konzentration der löslichen Phenole im Vergleich zu den anderen Behandlungen nicht unterscheidet. Somit zeigt sich, dass die terpenhaltigen Harze, die vermehrt in mit

Methyljasmonat behandelter Borke produziert werden, direkt für den beobachteten Rückgang der *I. typographus*-Kolonisation und Reproduktion verantwortlich sein könnten.

Um die Resistenz gegenüber dem weißen Kieferrüsselkäfer, *P. strobi*, zu untersuchen, wurde Jasmonsäure in die terminalen Abschnitte des Stammes von *P. abies*, die bevorzugten Fraßstellen des Insekts, injiziert. Die Behandlung hatte keine Auswirkung auf das Verhalten von *P. strobi*. Trotzdem bewirkten Jasmonsäure sowie Käferfraß, wie auch mechanische Verwundung Veränderungen im Terpengehalt des terminalen Stammes. Im Borkengewebe bewirkte das Fraßverhalten der Kieferrüsselkäfer und die mechanische Verwundung, jedoch nicht Jasmonsäure, einen zweifachen Anstieg des Gehalts an Monoterpenoiden und Sesquiterpenoiden. Beides hatte aber keine signifikanten Auswirkungen auf die Konzentration der verschiedenen Diterpene des Oleoresin. Sowohl Käferfraßverhalten als auch Jasmonsäure bewirkten in den Nadeln einen fast zweifachen Anstieg der Akkumulation von Monoterpenoiden und Sesquiterpenoiden und einen dreifachen Anstieg der akkumulierten Diterpene 32 Tage nach Behandlungsbeginn. Mechanische Verwundung zeigte keinen Effekt. Obwohl das Käferfraßverhalten den Terpengehalt verändert, haben diese Veränderungen keine Auswirkung auf die Abwehr unter den getesteten Bedingungen.

Um die Rolle von jasmonatabhängigen Signalwegen auf die Entwicklung induzierter Abwehrmechanismen der Norwegischen Fichte zu untersuchen, wurden Zellkulturen verwendet, die ein experimentell besser handhabbares und gut definiertes System zur Untersuchung dieser lokalisierten Abwehr bieten als ausgewachsene, voll entwickelte Bäume. Diese *P. abies*-Zellsuspensionskulturen produzieren konstitutiv *de novo* kleine Mengen verschiedener Monoterpen-Hydrokarbone, deren Produktprofil denen ausgewachsener Bäume entspricht. Es konnte keine Akkumulation von Sesqui- und Diterpenen festgestellt werden. Dennoch zeigte die wiederholte Anwendung von Methyljasmonat oder einem fungalen Elicitor eine dreifache Akkumulation von Monoterpenen. Die Messungen der Monoterpensynthaseaktivität, der entscheidende Schritt in der Monoterpen-Biosynthese, zeigte, dass die Enzymaktivität sowohl durch den fungalen Elicitor als auch durch Methyljasmonat signifikant induziert wurde, wobei Methyljasmonat den größten Effekt hervorrief. Diese Ergebnisse bestätigen, dass der

Jasmonat-Signalweg einen wichtigen endogenen Regulator induzierter Terpenbiosynthese in Fichten darstellt.

Zusammenfassend weisen die Ergebnisse dieser Studie darauf hin, dass induzierte Abwehr, wie die Bildung von Wundharzgängen, die Verstärkung des Harzflusses und die vermehrte Anhäufung von volatilen Terpenen und diterpenen Harzsäuren, ausgelöst durch den Einsatz von Jasmonaten, eine wesentliche Rolle bei der Abwehr verschiedenster Fraßfeinde der Norwegischen Fichte spielt. Die Ergebnisse bestätigten weiterhin, dass der Stoffwechselweg von Jasmonaten direkt die Bildung dieser induzierten Abwehr reguliert.

Obwohl die dargestellten Ergebnisse miteinander korrelieren, sind weitere Untersuchungen notwendig, um die tatsächlichen Abwehrrollen dieser Metabolite darzustellen. Dabei kann nicht ausgeschlossen werden, dass die Resistenz, die durch die Jasmonatbehandlung hervorgerufen wird, das Ergebnis anderer Abwehrstoffe als der Terpene darstellt. Die Spezifität von Jasmonaten zu einer oder anderen Klasse von Abwehrstoffen ist bei Koniferen noch nicht gezeigt worden. Zunehmendes Wissen der Biochemie und der molekularen Biologie der Abwehrmetabolismen machen es heute leichter, die Gene zu identifizieren, die die Bildung einzelner Abwehrstoffe oder Klassen von Abwehrstoffen in Koniferen regulieren. Solche Gene können verwendet werden, um transgene Koniferen mit verändertem Abwehrprofil herzustellen.

Stichwörter: Abwehrmechanismus, Borkenkäfer, *Ceratocystis polonica,* Elicitor, Fichtenborkenkäfer, induzierte Abwehr, *Ips typographus*, Konifere, mechanische Verwundung, Methyljasmonat, Monoterpene, Oktadekanoidweg, Phenole, Phloem, *Pissodes strobi,* weißer Kieferrüsselkäfer, Wirt-Besiedlungs-Sequenz, Wundharzgänge, Zellsuspensionkultur.

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LIST OF PUBLICATIONS AND SCIENTIFIC PRESENTATIONS

Book Chapter:

1. Schmidt, A., **Zeneli, G**., Hietala, A.M., Fossdal, C.G., Krokene, P., Christiansen, E., and J. Gershenzon. **2005.** "Induced chemical defenses in conifers: Biochemical and molecular approaches to studying their function". *In:* Romeo, J.T. (ed): Chemical Ecology and Phytochemistry in Forest Ecosystems. Vol. 39. Elsevier, Amsterdam. pp. 1-28.

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